WHERE THE LAND MEETS THE SEA

FOURTEEN MILLENNIA OF HUMAN HISTORY AT HUACA PRIETA, PERU

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APPENDIX 17

ESTIMATING HAPLOGROUP AFFILIATION THROUGH ANCIENT MTDNA ANALYSIS FROM THE HUACA PRIETA BURIALS

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INTRODUCTION

A total of 21 dental samples from 21 individuals from Huaca Prieta (and Paredones) underwent ancient mtDNA analysis in an attempt to establish haplogroup affiliation and address questions about biological relatedness among the burial population. Dillehay's research team excavated 11 of the samples (10 from Huaca Prieta and 1 from Paredones), and another 10 samples from Huaca Prieta were obtained from Bird's collection at the American Museum of Natural History (AMNH) in New York City. Jessica Blair at the Vanderbilt University Medical Center and Center for Human Genetics and Raúl Tito at the University of Oklahoma Laboratory for Molecular Anthropology and Microbiome Research attempted to extract and amplify the ancient mtDNA from those samples. The process of analysis and the results are detailed below. Although our study did not result in any individuals being assigned to a Native American haplogroup, we present the phases of analysis and the specific mutations that were observed in particular samples.

CODING SYSTEM

The samples exported from Peru are referred to as HP1, HP2, HP3, and so forth; the samples from the AMNH are referred to as AMNH1, AMNH2, and so forth; and the single sample from Paredones is coded as PAR1 (see table A17.1).

In the section that provides specific information on amplification and sequencing, the coding system is as follows:

- The first portion is the sample ID (a numeral in front of letters indicates 2nd or 3rd extract);
- The 2nd portion is the fragment "chunk" (1st, 2nd, 3rd, 4th);
- The 3rd portion is the primer used for that specific fragment's data.

Example: 2HP3-1-15986 = 2nd extract of HP3, 1st fragment, primer 15986.

Table A17.1. Summary data on the Huaca Prieta mtDNA samples exported from Peru

Sample no.	*Arch. code*	*Museum code*	*Tooth*	*Sex*	*Age (years)*	*Extraction 1*
HP-1	Burial 1		Md RM2	F	Adult	failed
HP-2	Burial 1		Md RM2	M	35–45	failed
HP-3	Burial 3		Mx LM2	F	35–45	failed
HP-4			Mx RM1	?	12–15	failed
HP-5	Burial 2		Md RM3	F	50–60+	failed
HP-6	Burial 1		Mx RM2	F	Adult	failed
HP-7	Burial 6		Mx RM3	M	18–25	failed
HP-8	Burial 4		Mx RM2	?	3–5	failed
HP-9	Burial 1		Md RM1	?	6–10	failed
HP-11			Md RM1		7–8	amplified when incubated for longer time
PAR-1			Md RM 1 or 2			chelexed; failed
AMNH1	7-2-2	99.1/890	Mx RM2			failed
AMNH2	3-D3	99.1/896	Md RM3			failed
AMNH3	3-F	99.1/897	Mx LM3			chelexed; failed
AMNH4	3J	99.1/900	Mx LM3			chelexed; failed
AMNH5	3-0-3	99.1/901	Md LM2			chelexed; failed
AMNH6	3-0-2	99.1/902	Md RM3			chelexed; mutations at 16129A, 16169T, 16173T
AMNH7	3-0-1	99.1/903	Md RM3			chelexed; mutation at 16266T and 16223T
AMNH8	2-G1-2	99.1/906B	Md LM3			chelexed; failed
AMNH9	2-G2-4	99.1/908	Md RM3			chelexed; mutation at 16266T and 16129A.
AMNH10	3-T	99.1/904	Md RM2			chelexed; amplified

RESULTS

The preliminary ancient mtDNA testing of these samples shows that 62% of the samples that were tested (13 out of 21) have mtDNA, albeit in low counts and in low quality. Additional techniques to clean the samples and increase the quantity may result in more successful identification of haplogroups and sequence data, so that questions about biological affiliation among the individuals at Huaca Prieta can be addressed.

The initial attempt to obtain mtDNA from samples HP1 through HP9 yielded no results (fig. A17.1a).

The second attempt (fig. A17.1b) tested whether or not samples HP1–HP5 were inhibited (that is, whether some material in the sample such as metal ions was inhibiting the mtDNA from amplifying). Results showed that HP2 was indeed inhibited. This was demonstrated by combining HP2 with a well-preserved ancient sample with a known haplogroup (MC5, Malata Chullpa sample 5 from the site of Malata in southern Peru), and the HP2 sample did not

Sample no.	*Extraction 2*	*Extraction 3*	*Unit*	*Floor*	*Layer*	*Pit*	*Wall*
HP-1	chelexed; not Hg D; mutation at 16129A and 16294T				15	3	W
HP-2	chelexed; sample inhibited and contaminated; no data		3	7			
HP-3	chelexed; not Hg B	not Hg A, B, C, or D; mutations at 16311C and 16362C	16		5		
HP-4	chelexed; no data.		16		2		
HP-5	chelexed; not Hg A; mutations at 16362C, 16266T, 16129A, and 16080G		16		2		
HP-6	chelexed; mutation at 16129A; this is seen in Hg A5c, A7, A9; B4c1a1a, B5a2; C4a1; Z1, D2, D4a		10				
HP-7	chelexed; mutation at 16044C; possible contamination		16				
HP-8	chelexed; failed		16		2		
HP-9	chelexed; mutation at 16325C and 16223T	contamination from J. Blair	23		7		
HP-11	failed		16	NW muro	2		
PAR-1	chelexed; failed						
AMNH1	chelexed; mutations at 16295T and 16319A						
AMNH2	chelexed; mutation at 16111T						
AMNH3	chelexed; failed						
AMNH4	chelexed; mutation at 16223T						
AMNH5	chelexed; failed						
AMNH6							
AMNH7							
AMNH8	chelexed; failed						
AMNH9							
AMNH10	mutations at 16261T and 16304C						

amplify. There is no band in the gel in the right lane of HP2, thus confirming inhibition (fig. A17.1b). It is unknown what is inhibiting the mtDNA, but salt is a likely factor among other possible inhibitors, such as metal ions.

Chelex resin was then added to the samples to increase the likelihood of mtDNA amplification. Chelex resin "cleans" the sample; they are resin beads that remove inhibitory metal ions remaining in the DNA solution, while leaving the DNA intact so that amplification is possible. The addition of Chelex to each sample led to some promising results; faint bands appeared in lanes 2 and 3 (fig. A17.1c). Given the likelihood that these samples contained some mtDNA, they were sent to the Sequencing Core at the Vanderbilt University Medical Center for sequencing.

Although one of the tests on HP3 suggested that it had the 9-base pair deletion characteristic of Haplogroup (Hg) B (fig. A17.1a), the sequence data from HP3 did not show the 9-base pair deletion, suggesting that the individual does *not* belong to Hg B. Subsequent runs were unable to obtain data for any of the known Native American haplogroups

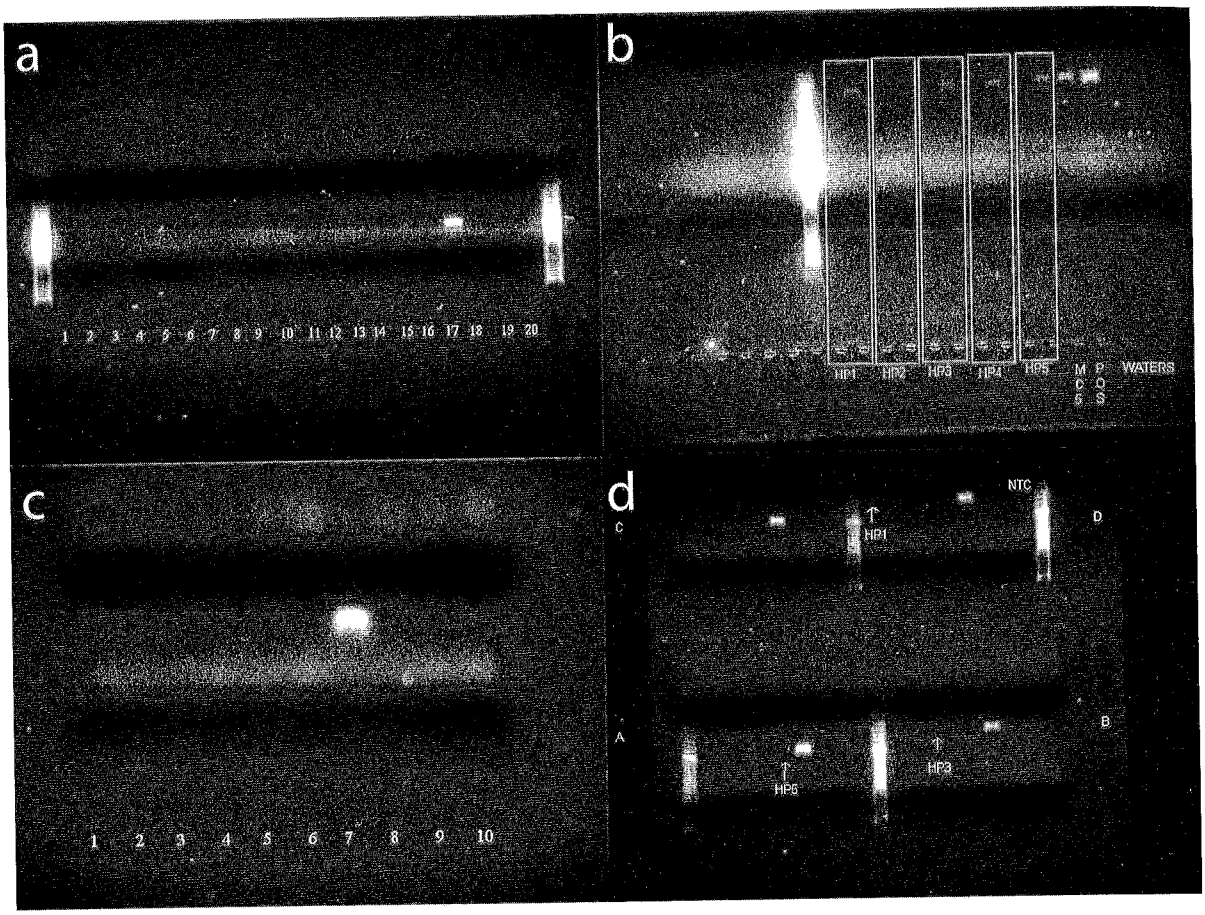

FIGURE A17.1. DNA results from Huaca Prieta tooth studies: *a*, initial attempt to obtain mtDNA from HP samples 1–9 (lanes 1–9) post–DNA extraction; the bright band is positive modern control (lane 17); no HP samples had any indication of amplification, nor did any negative controls (lanes 16, 18–20); *b*, inhibition testing for HP1–HP5; within each box, the left lane contains the HP sample only and the right lane contains the HP sample plus positive ancient DNA (MC5); a diminished or no band in the right lane indicates inhibition of the HP sample, as compared to the lane marked MC5: HP2 has no band in the right lane and thus is inhibited; the bright band on the far right is the positive modern control; *c*, image of a gel showing the faint bands in lane 2 (sample HP2) and lane 3 (sample HP3); the bright band is the positive control (lane 7); lanes 8–10 are negative controls; *d*, image of gel showing bands from HP1, HP3, and HP5; letters correspond to the haplogroups being tested for in that set; bright bands are positive modern controls; lanes to the right of the positive control are negative controls.

(Hg A, C, or D) (fig. A17.1d). The inability to identify one of the traditional Native American haplogroups (A, B, C, D) (Schurr 2004) in HP3 could suggest that it is a contaminated sample. There is also a very low possibility that HP3 belongs to haplogroup X or a heretofore unknown haplogroup in ancient South America. In sum, HP3 is *not* Hg B; nor could it be confidently assigned to Hg A, C, or D.

As seen in figure A17.1d, after Chelex cleaning, HP1 was amplified for haplogroup D and HP5 was amplified for Hg A. However, sequencing data from these amplifications indicate that neither belongs to the respective haplogroup that each was amplified in. That is, HP1 is *not* Hg D, and HP5 is *not* Hg A.

All of the HP samples underwent three extractions, described in more detail below. Among the AMNH samples, AMNH1–AMNH3 underwent two extractions, AMNH4 and AMNH5 went through three extractions because the first extraction failed, and AMNH6–AMNH10 underwent two extractions. PAR1 went through two extractions.

The specific details of the amplification and sequence process for each sample are presented below.

HP1 (Adult female, Sample Mand RM2): The first amplification yielded no results. It was then chelexed, and the sample amplified. This individual does *not* belong to Hg D. Sequence data show that on the first fragment (primer 15986) a mutation is present at 16129A [2HP1-1-15986 (16153frag rCRS).[1] There is also a mutation at 16294T. This sample was subsequently examined by the Laboratory for

1. rCRS = Revised Cambridge Reference Sequence.

Molecular Anthropology and Microbiome Research at the University of Oklahoma, which concluded that there was not enough mtDNA preserved for analysis.

HP2 (35–45 yr old male, Sample Mand RM2): The first amplification yielded no results. It was then chelexed, but the sample remained inhibited, so the haplogroup is unknown. The second attempt at amplification of the third fragment (primer 16355) shows that the sample was contaminated by the lab technician, Jessica Blair. The one exception is at 16278T, which is not from Blair [2HP2-3-16355]. Although all precautions were taken to prevent modern contamination, Blair's mtDNA contaminated a sample in two or possibly three cases, which are always noted.

HP3 (35–45 yr old female, Sample Max LM2): The first amplification yielded no results. It was then chelexed, and the subsequent sequence data showed that this individual does not belong to Hg A, B, C, or D. It is likely that this sample is contaminated, with a low possibility that it belongs to Hg X or some other unknown haplogroup in South America. Sequence data show that the 4th fragment (primer 16232) has two mutations: 16311C 16362C [3HP3-4-16232]. Sequence data of the 4th fragment (primer 16404), also showed the mutation at 16311C.

HP4 (12–15 yr old, sex unknown, Sample Max RM1): the first amplification yielded no results. It was then chelexed, but no mtDNA was retrieved.

HP5 (50–60+ yr old female, Sample M and RM3): The first amplification yielded no results. It was then chelexed, and the sample amplified. This individual does not belong to Hg A.

Sequence data show that on the 4th fragment (primer 16232), signs of a mutation (double peak) are found at 16362C [2HP5-4-16232], and on the 4th fragment (primer 16404) there is a sign of a mutation at 16266T. Also, the first fragment (primer 15986) shows signs of a mutation at 16129A [2HP5-1-15986], and both primers for the first fragment show signs of a mutation at 16080G [2HP5-1-both].

HP6 (Adult female, Sample Max RM2): The first amplification yielded no results (no mtDNA was retrieved). The sample was then chelexed, and it amplified. The sequence data showed that on the first fragment (primer 15986) there was a sign of a mutation at 16129A [2HP6-1-15986].

HP7 (18–25 yr old male, Sample Max RM3): The first amplification yielded no results (no mtDNA was retrieved). The sample was then chelexed, and it amplified. The sequence data showed that on the first fragment (both primers) there was a sign of a mutation at 16044C [2HP7-1-both]. This mutation is not observed in Hg's A, B, C, D, or X, so this sample may be contaminated.

HP8 (3–5 yr old, sex unknown, Sample Max drm2): The first amplification yielded no results (no mtDNA was retrieved). The sample was then chelexed, but there was no amplification, so the haplogroup is unknown.

HP9 (6–10 yr old, sex unknown, Sample Mand RM1): The first amplification yielded no results (no mtDNA was retrieved). It was then chelexed. The sequence data show a sign of a mutation at 16325C on the 3rd fragment (primer 16190) [2hp9-3-16190] and a sign of a mutation at 16223T on the 3rd fragment (primer 16355) [2hp9-3-16355]. The sequence data obtained from the third extraction showed contamination from Jessica Blair (16192T).

HP10: This code was used for a carbon sample that was radiocarbon dated.

HP11 (5–9 yr old, sex unknown, Sample Mand RM1): The first extraction amplified, but subsequent attempts to get sequence data failed.

PAR1 (Site of Paredones, Adult, sex unknown, Sample Mand RM): The first and second extractions were chelexed, but both failed to amplify. No data on haplogroup.

THE AMNH SAMPLES

AMNH1 (99.1/890): The first extraction did not amplify. The second extraction was chelexed, and the sample amplified. This second extraction was sequenced and the 2nd fragment (primer 16190) had mutations at 16295T and 16319A [2amnh1-2-16190]. Also, the 2nd fragment (primer 16355) showed a mutation at 16295T [2AMNH1-2-16355]. This sample was also examined by the Laboratory for Molecular Anthropology and Microbiome Research at the University of Oklahoma, which concluded that there was not enough mtDNA preserved for analysis.

AMNH2 (99.1/896): The first extraction did not amplify. The second extraction was chelexed, and the sample amplified. This second extraction was sequenced, and the 1st fragment (both primers) showed mutation at 16111T [2AMNH2-1-both].

AMNH3 (99.1/897): The first and second extractions were chelexed, but both failed to amplify. No haplogroup data were obtained. This sample was also examined by the Laboratory for Molecular Anthropology and Microbiome Research at the University of Oklahoma, which concluded that there was not enough mtDNA preserved for analysis.

AMNH4 (99.1/900): The first extraction was chelexed, but it failed to amplify. The second extraction was also chelexed, and it amplified. This second extraction was sequenced. The third fragment (primer 16355) showed a sign of a mutation at 16223T [2AMNH4-3-16355], and the third extraction (primer 16404 and both primers) showed that it matched the rCRS.

AMNH5 (99.1/901): The first extraction was chelexed, but it failed to amplify. The second extraction also failed.

AMNH6 (99.1/902): The first extraction was chelexed, and it amplified. This first extraction was sequenced, and the 2nd fragment (primer 16251) showed mutations at 16129A, 16169T, and 16173T. A fourth mutation at 16192T could be contamination from Jessica Blair [AMNH6-2-16251]. This sample was also examined by the Laboratory for Molecular Anthropology and Microbiome Research at the University of Oklahoma, which concluded that there was not enough mtDNA preserved for analysis.

AMNH7 (99.1/903): The first extraction was chelexed, and it amplified. This first extraction was sequenced. The 3rd fragment (primer 16190) showed a mutation at 16266T [AMNH7-3-16190] and the 3rd fragment (primer 16355) showed mutation at 16223T and 16266T [AMNH7-

3-16355]. The fourth fragment (primer 16404) also showed a sign of a mutation at 16266T [AMNH7-4-16404].

AMNH8 (99.1/906B): The first and second extractions were chelexed, but both failed to amplify, so no data were obtained on the haplogroup designation.

AMNH9 (99.1/908): The first extraction was chelexed, and it amplified. This first extraction was sequenced, and the 3rd fragment (both primers) showed signs of a mutation at 16266T [AMNH9-3-both]. The 1st fragment (primer 15986) showed signs of a mutation at 16129A [AMNH9-1-15986]. This sample was also examined by the Laboratory for Molecular Anthropology and Microbiome Research at the University of Oklahoma, which concluded that there was not enough mtDNA preserved for analysis.

AMNH10 (99.1/904): The first extraction was chelexed, and it amplified. The second extraction also amplified, and sequence data from the second extraction of the 3rd fragment (primers 16190 and 16355) showed mutations at 16261T [2AMNH10-3-16190] and 16304C [2AMNH10-3-16355].

DISCUSSION AND CONCLUSIONS

The inability to replicate the results in a second lab is cause for concern, though it should be noted that the University of Oklahoma Lab did not chelex samples, which may explain at least some of the failure to amplify the mtDNA. Although every additional step in the analysis introduces possibilities for contamination (such as the chelex step), the clean controls suggest that those run by the Center for Human Genetics Research Laboratory at Vanderbilt University may have yielded accurate albeit incomplete results. Thus, while some specific mutations were documented, none of the samples could be assigned to a specific Native American haplogroup.

BIOLOGICAL KINSHIP UNKNOWN

Based on the archaeological context of the human burials, we were interested in establishing whether or not individuals from Unit 16 shared the same maternal lineage (Samples HP 3, 4, 5, 7, 8, and 11). The poor DNA preservation prevented the identification of haplogroups, however, so this particular question could not be addressed. But the sequence data did reveal that HP3 (adult female) and HP5 (adult female) both have the same mutation at 16362C. This mutation is observed in haplogroups/haplotypes A2, A4, A4d, A6, B4b1b, and D.

Additionally, we investigated whether the individuals from separate burial contexts (separate units) were more distantly related than those found in proximity (in the same units). There were three individuals from units other than Unit 16: Units 3, 10, and 23. The mtDNA from the adult (HP2) in Unit 3 was inhibited, so his haplogroup is unknown and could not be compared to different units.

One mutation (16129A) was shared by HP1 (adult female), HP5 (adult female), HP6 (adult female), and AMNH6 (sex unknown). Although the goal was to evaluate whether females were closely related, as might be expected in matrilocal societies, this question could not be addressed because this particular mutation is seen in all four Native American haplogroups: A, B, C, and D. Thus, while those individuals appear to belong to one of the traditional Native American haplogroups, it does not reveal whether they are closely related on the maternal line. This hypothesis clearly requires further testing. It is unknown whether or not the males at Huaca Prieta were closely related, because only one male provided sequence data.

HP5 and AMNH9 share two mutations: 16266T and 16129A. Mutation 16266T is seen in Hg D5a2, and no other Native American haplogroups, but it should be accompanied by 1438A, which was not tested for in these samples. Mutation 16129A is seen in all haplogroups. Within Hg D it is specifically seen within D2 and D4a. The particular mutation (16266T) was further shared by AMNH7 (HP5 and AMNH9 also have it).

Granted, there is much biological homogeneity in the Andes (Kemp et al. 2009; Lewis et al. 2007; Shinoda et al. 2006), so specific haplogroup designations and additional sequence data are needed before any claims of maternal relatedness can be made among the Huaca Prieta burials.

HP7, a young adult male, has a mutation 16044C, but none of the other 11 burials with sequence data showed that particular mutation. Moreover, mutation 16044C is not typically seen in any of the Native American haplogroups (A, B, C, D, X), so this sample may be contaminated, but it is not contamination from Jessica Blair.