Author: Frederika Kaestle <frederika.kaestle@yale.edu> at np--internet
Date: 9/18/00 9:51 PM
Priority: Normal
CC: FP McManamon at NP-WASO-DCA
TO: Jason Roberts at NP-WASO-DCA
Subject: Re: Draft DNA Report Comments

Jason,

I am attaching my report in both Rich Text Format (which should include the tables and figures in the correct format) and in MS-DOS text (which doesn't). I'm also attaching separate versions of the tables in both Mac and PC format, and a revision of the Gell Image Log that includes three additional gels. I will email you TIF copies of those tomorrow. The figures are identical to the original version I sent you. I will also mail you hard copies of the report tomorrow. I apologize for the delay in getting this revision to you. I had some problems getting a primer shipped to me that I needed to complete the report. In revising my report I have

1. Added a section on my laboratory facilities

2. Expanded the section describing PCR inhibitors

3. Fixed the typo in the figure legend

4. Expanded my logic on the contamination issue, and included some new data on this issue

5. Discussed the correlation between using high-concentration extracts and detecting contamination

6. Included the GeneReleaser protocol methods in the appendix, and incorporated a discussion of the final round of testing in the body of the report

7. Set up my report as a separate document rather than a letter

8. Added a table (table 1) depicting the mutations defining the haplogroups A, B, C, D and X (found in modern and ancient Native Americans) and I (which I belong to).

9. Revised my Gel image log to add three images from the final round of testing

Let me know if you have any questions or need me to make any further changes.

Frederika

> Frederika:
>
> Thanks for the fine draft Kennewick DNA report, dated August 10, 2000, you completed. We have the following comments for the final DNA report. If these are unclear or you disagree with them, please call me at 202-343-4105 or Jason Roberts at 202-343-1010.
>
> General Comments:
>
> This report is a very thorough and clearly written recording of the methods that you undertook in the analysis of the two DNA bone samples [97-1-16(MCa) and 97-1-12b(13)frag] from the Kennewick human remains.
>
> Specific Comments:
>

1. In order to provide greater detail about the process of analyzing ancient DNA, we would appreciate a fuller description of your laboratory's facilities (physically isolated work area, separate/devoted ancient DNA equipment, etc.).

2. Additionally, we believe a general paragraph describing DNA (PCR) "inhibitors" (i.e., human induced or naturally occurring) and whether you detected any when performing your investigation on the Kennewick bone samples would be very informative.

3. Typo - an extra period appears at ". Lane 17: amplification negative control.. Note presence." on page 8.

4. Please address in greater detail issues regarding "contamination" and the methods you applied to control for and recognize DNA contaminants. In particular, you stated that "[t]hese results are consistent with a very low level of contamination of the extracts with modern DNA, perhaps from the investigator or originating at the reagent or lab disposables manufacture" (see pg. 6). Could you further describe the ancient DNA contamination issues related to this statement (i.e., can you be more specific about the contaminant's origin?

5. Please describe more fully why you are certain it is modern DNA contamination in the extracts - see pg. 9) - especially detailing the use of high concentrations of extract and the correlation, if any, with the amplifying non-ancient DNA.

6. Please describe the protocols and methods you utilized during your final round of testing.

7. Please provide your final report a title and set it up as a separate document, rather than as the body of a letter.

A marked-up copy of your draft DNA report also will be faxed to you to help in the preparation of the final report.

Thank you,

Frank

Report on DNA analysis of the Remains of "Kennewick Man" from Columbia Park, Washington

Frederika Kaestle

Molecular Anthropology Laboratory, Department of Anthropology, Yale University, New Haven, CT 06520

Introduction

This report details the ancient DNA analyses for two bone samples [97-1-16(MCa) and 97-1-12b(13)frag] from the human remains commonly referred to as 'Kennewick Man' or 'Ancient One', which were received by Dr. Frederika Kaestle (FAK) on May 19, 2000. In laboratory records these are referred to as 'Kennewick Metacarpal' or 'Kenn M-C" and 'Kennewick Rib' or 'Kenn Rib' respectively. In brief summary, four DNA extractions

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were attempted on each sample. Results of amplification attempts are most consistent with the inability to extract ancient DNA from these samples using available technology.

Due to the sensitivity of the study of these remains, digital images were taken during the extraction procedures to document the protocol, and copies on a zip disk were submitted to the Department of the Interior (DOI), as were hard copies of the electrophoretic gel images. Detailed extraction and amplification protocols may be found in appendix A. All extractions and amplifications were performed by FAK, primary investigator (8 years experience with ancient DNA).

Ancient DNA: Background

DNA, the genetic material contained in our cells, is normally protected from the majority of damaging forces such as hydrolysis, oxidation, chemical damage, and ultra-violet radiation by our living bodies, and the majority of damage that does occur is corrected by the activities of proofreading enzymes and other mechanisms in the cell. However, upon death these protections and repair mechanisms break down, and the DNA quickly degrades into small fragments with frequent sites of molecular damage. Thus, although we can extract DNA from the remains of deceased individuals, the analysis of this genetic material is subject to a multitude of problems that are not issues when analyzing modern DNA. First, because the DNA has degraded, very few intact copies of particular regions of the genome remain for analyses. Only the extremely sensitive methods of the Polymerase Chain Reaction (PCR), a process that allows us to make millions of copies of a target fragment of DNA, makes ancient DNA analyses possible. In addition, although this process allows the amplification of long target fragments from high-quality modern DNA (thousands of nucleotide bases long, in some cases), the fragmentary nature of ancient DNA results in the inability to amplify fragments of ancient DNA longer than approximately 200-300 nucleotide bases in most cases. This limits the type of genetic tests that can be applied to ancient DNA to those that do not require long intact fragments of DNA (for example, the traditional 'DNA fingerprint' technique, which involves digesting long fragments of DNA with various restriction enzymes and exposing them to a DNA probe, would not be feasible). The low number of copies and degraded state of those copies creates an additional problem for ancient DNA research, that of contamination. Because modern DNA is of superior quality, in the sense that the enzymes utilized in genetic analyses have evolved or been designed by humans to interact with long fragments of undamaged DNA, and because modern DNA simply exists in higher concentration, the presence of even small amounts of modern DNA (shed skin cells, cells from the mouth in aerosolized saliva, follicles from shed hair, etc.) in or on laboratory equipment and reagents can easily overwhelm the small amount of ancient DNA extracted from deceased individuals. Thus, every measure to eliminate the possibility of such contamination, and to detect it when it (almost inevitably) occurs, is required for ancient DNA research. The replication of ancient DNA work in multiple laboratories is also helpful in detecting contamination, as it is extremely unlikely that identical DNA contamination will occur in two separate laboratories.

A final problem that complicates ancient DNA analyses has little to do with the extracted DNA, but actually involves other factors, which are coextracted with the DNA from the sample. In general ancient DNA is extracted from tissue, mostly bone and teeth, which has been exposed to various environments for long periods of time. Often the bones absorb factors from the surrounding soil matrix such as humic acids, or byproducts of fungus or bacteria living in the soil. In addition, byproducts of the breakdown of the soft tissue, such as Maillard products, can be absorbed. Many of these factors will coextract with DNA (that is, they are not eliminated during the extraction procedure), and interfere with the functions of enzymes used to amplify the DNA during PCR (e.g., Taq polymerase). The inhibition of the Taq polymerase during PCR results in little to no amplification of the target ancient DNA fragment, and the appearance that the extract contains no DNA. Therefore, it is important to eliminate inhibition as a possible cause of a false negative result. This can be done in several manners. During many PCR reactions some of the primers (short fragments of DNA added to the reaction to target a specific region of the genome) may bind to each other and be amplified by the Taq polymerase, producing a short fragment (usually 40 to 80 nucleotide bases long) usually called primer-dimer. Therefore, the presence of primer-dimer indicates the proper activity of the Taq polymerase, even in the absence of amplification of the target fragment of ancient DNA.

Laboratory Facilities

The molecular anthropology laboratory facilities at Yale are located in two physically separate laboratory spaces, with separate air systems. Each room is regularly decontaminated with bleach, and ultra-violet (UV) lights mounted in the ceilings are used each evening as an additional measure to destroy DNA. The laboratory equipment [including fume hood, bio-safety cabinet, refrigerators, freezers (both -20 and -80 degree), centrifuges (including vacuum and refrigerated), Ultra-Violet cross-linker, water baths, heating blocks, incubators, vortexers, rockers, pH meters, balances, vacuum pump, dremel tool, thermal cyclers, vertical and horizontal electrophoresis rigs, microwave, digital imaging system, and electronic and manual pipetors] are dedicated equipment that are never transferred between laboratories and are regularly bleach and/or UV decontaminated. All disposable products such as pipette tips and microcentrifuge tubes are either purchased DNA-free or of similar quality (e.g., Dnase, Rnase and protease free), or are decontaminated prior to use, as are laboratory reagents. Water is purchased in DNA and HPLC grade and often additionally filtered through a 0.2 um vacuum filter. Laboratory reagents are purchased and prepared in small volume to minimize opportunities for contamination during use. Laboratory personnel wear protective gear including laboratory coats, latex gloves, shoe covers, hair covers and face masks to prevent contaminating the ancient DNA laboratory with their own DNA, or with amplified DNA from our own or other laboratories. Ancient DNA extraction and the preparation of amplification reactions are performed in one of the laboratories, while the actual amplification procedure (PCR) and subsequent analyses are performed in the second laboratory. Automated sequencing of amplified and sequenced DNA is performed in a third laboratory located in a separate building on a shared ABI 377 Automated

Sequencer. Data analyses are performed using a Mac G4 and a Dell PC with a Pentium III processor, located in FAK's office.

Extraction

Detailed ancient DNA protocols may be found in Appendix A. The following summarizes the application of these protocols to the Kennewick samples in particular.

The accession number of the rib sample was removed with a razor blade before analysis. The metacarpal fragment did not have an accession number on it. Surfaces of both bone samples were cleaned with 10% bleach and UV irradiated to eliminate modern DNA contaminants. Bone powder was removed from internal surfaces of the bone fragments using a Dremel tool. In both cases the bone fragments were quite brittle and easily broken, generally indicative of poor preservation of organics. The presence of soil matrix within each sample was noted, and was particularly prevalent in the rib sample. Between 0.124 and 0.358 grams of bone powder was utilized in each extraction. In three of the extraction procedures the bone powder was decalcified over the course of three days using 0.5 Molar EDTA, pH 8.0, with EDTA negative controls added each day. In the fourth extraction, to address the possibility that ancient DNA was being lost during the EDTA washes, no decalcification was performed. In all cases the powdered bone and negative controls were digested using proteinase K overnight at 55 degrees Celsius. It was noted that after digestion a significant portion of the powdered bone remained, which is typical of samples that are heavily mineralized, as was the case with these samples (Powell and Rose 1999). Under optimal conditions (i.e., little to no mineralization) decalcified bone powder will be completely digested by proteinase K.

In one extraction, the digested bone powder and negative controls were filtered and concentrated using Centricon-100 filtration units (Millipore™), and silica extracted using the QiaQuick PCR Purification Kit (Qiagen™) (Yang et al.1998). In a second extraction the digested bone powder and negative controls were phenol-chloroform extracted and then filtered and concentrated using Centricon-100 filtration units. In a third extraction a small portion of the Centricon-100 filtered digested bone powder and negative controls were extracted according to the manufacturer's protocols using GeneReleaser (BioVentures™), a proprietary product designed to combine DNA extraction and amplification. In the fourth extraction, the digested but non-decalcified samples and negative control were filtered and concentrated using Centricon-100 filtration units and silica extracted using the QiaQuick PCR Purification Kit. Attempts were made to amplify short fragments of ancient DNA from each extraction using primers designed for ancient DNA analysis (see table 1 for primers and annealing temperatures). In cases of amplification inhibition by co-extracted PCR inhibitors (see above) it is sometimes possible to overcome inhibition through dilution. Therefore, all sample extracts except the GeneReleaser extracts were diluted to 1:10 and 1:50 concentrations and amplified. On occasion additional digestion of contaminating proteins after extraction can also overcome PCR inhibition. For this reason a small portion of the first (silica) extracts were re-digested with proteinase K in the presence of Tween-20 and amplified. Finally, amplifications were attempted using three of the extractions (excluding the GeneReleaser

extract) in very high concentrations (approximately four times the usual concentration) in an attempt to compensate for possible low to absent concentration of ancient DNA. All amplifications were performed with high concentrations of Bovine Serum Albumin, which has been shown to reduce the affect of PCR inhibitors often co-extracted with ancient DNA. Negative amplification controls were utilized in all cases, and positive controls (consisting of modern DNA from the investigator, extracted in the post-PCR laboratory) were utilized in amplifications involving high concentrations of extract. In some cases, amplifications were exposed to DNAse I before primers and extracted DNA were added, a procedure that has been shown to eliminate contaminating DNA from the PCR reagents (Eshleman and Smith 2000). PCR products were separated on agarose gels through electrophoresis, stained with ethidium bromide, and visualized under UV light. Digital images of the electrophoretic gels were taken.

Amplification

Initial efforts focussed on amplifying mitochondrial DNA (mtDNA) rather than nuclear DNA (nDNA). As discussed above, because DNA degrades over time due to the effects of temperature, hydrolysis, radiation, oxidation, and acidity, ancient samples often possess only highly damaged DNA (Herrman and Hummel 1994). MtDNA is located not in the nucleus but rather in the mitochondria, the cellular organelles responsible for energy production. Most cells possess hundreds of mitochondria, each with several copies of the mitochondrial genome. MtDNA is therefore present in many more copies than nDNA, and the likelihood that a particular fragment of mtDNA will survive over time is therefore much greater than the likelihood that a particular fragment of nDNA will. In addition, mtDNA is inherited only through the female line (i.e., mothers pass their mtDNA on to all of their children but fathers pass no mtDNA on). This allows us to identify maternal lineages of mtDNA united by their possession of a set of common mutations. All individuals possessing a particular mtDNA lineage can theoretically trace their maternal ancestry back to a single woman. Many mtDNA lineages, commonly called haplogroups, are limited in distribution among modern humans (but note that some are present in a wide range of modern peoples). Studies of modern individuals who are maternally Native American have shown that the majority possess mtDNA belonging to one of five maternal lineages, or haplogroups, called A, B, C, D, and X (e.g., see Smith et al. 1999; Lorenz and Smith 1996; Merriwether et al 1995; Schurr et al. 1990), defined by the possession of the diagnostic mutations seen in table 1. Although most modern Native American populations studied to date possess at least four of these haplogroups, haplogroup frequencies vary significantly between many groups, and some groups completely lack several of these haplogroups. In some cases, it appears that populations living within a restricted geographic region (such as the American Southwest) share similar frequencies of these haplogroups, while in other cases it appears that populations speaking related languages (such as the Algonquians) share similar haplogroup frequencies (Lorenz and Smith 1996; Malhi et al. 2000). In addition, within haplogroups particular single nucleotide mutations often occur in a relatively restricted area or population (Kaestle 1998; Schurr et al. 1999). Studies of ancient mtDNA from pre-Columbian Native Americans have shown that the majority of these individuals also possessed haplogroup A, B, C, D, or X (e.g., see Kaestle et al. 1999; O'Rourke et al. 1999; Stone and Stoneking 1998). Haplogroups A through D are also found among

modern Asians, albeit in relatively low frequency, and haplogroup B is also found among modern Pacific Islanders (Hagelberg and Clegg 1993; Merriwether et al. 1996, 2000; Torroni et al 1993). The modern distribution of haplogroup X is less well characterized. Thus far it has been found in low frequencies in Europe and Western Asia, but a suite of additional mutations distinguish the American haplogroup X lineages from those in the Old World (Brown et al 1998; Smith et al. 1999).

It is important to note that the possession of one of these haplogroups simply indicates that an individual has maternal Native American or Asian ancestors. What proportion of these ancestors derived from Native American or Asian populations, and the identity of the individual's paternal ancestors, is unknown. In addition, the terms Native American, Asian, European, etc. are not meant to imply racial differences or groups, but are instead fluid geographic categories describing fuzzy boundaries that may have shifted throughout prehistory.

Table 1. Markers identifying the five common mitochondrial haplogroups found among modern Native Americans. A '+' indicates the presence of a restriction site, a "-" indicates the absence of a restriction site. 'np' = nucleotide position.

Haplogroup	HaeIII np 663	9 bp deletion	HindII np 13259	AluI np 13262	AluI np 5176	DdeI np 1715	AccI np 14465
A	+	-	+	-	+	+	-
B	-	+	+	-	+	+	-
C	-	-	-	+	+	+	-
D	-	-	+	-	-	+	-
X	-	-	+	-	+	-	+
I	-	-	+	-	+	+	-

Amplifications were performed using primers that flank regions of the mitochondrial genome known to contain mutations defining the five maternal lineages present among modern Native Americans and Asians (see table 2). The results of amplification attempts and restriction digests of positive amplifications are presented in table 3.

Table 2: Primers used for PCR amplification

Haplogp	Primer Label	Primer Sequence (5'-3')	Location1	Annealing temp.	Fragment Length	Restriction Site
A	590for	ACCTCTCAAAGCAATACACTG	590-611	55	175 base pairs	+HaeIII np663
	765rev	GTGCTTGATGCTTGTTCCTTTTG	765-743			
B	8195for	ACAGTTTCATGCCCATCGTC	8195-8215	55	122 bp	+ 9 bp deletion
	8317rev	ATGCTAAGTTAGCTTTACAG	8317-8297			
C	13236for	AATCGTAGCCGGCGCCACTTCA	13236-13257	58	74 bp	-HinclI np13259
	13310rev	GCTAGGTGTGGTTGGTTGATG	13310-13290			+AluI np13262
D	5099for	CCTAACTACTACCGCATTCCTA	5099-5120	55	111 bp	-AluI np5176
	5210rev	GGGTGGATGGAATTAAGGGTGT	5210-5189			
X	1631for	ACTTAAACTTGACCGCTCTGA	1631-1651	51	162 bp	-DdeI np1715
	1793rev	CCCTTGCGGTACTATATC	1793-1776			
X	14421for	CTGACCCCCATGCCTCAGGA	14421-14440	55	194 bp	+AccI np14465
	14612rev	CTAAGCCTTCTCCTATTTATGG	14591-14612			

1 Based on numbering of mtDNA genome 1-16569 (Anderson et al. 1981)

In all cases, except the amplification attempts using high concentrations of extracts, no amplification product was detected, indicative of either the absence of DNA in the extracts or the presence of a PCR inhibitor that co-extracted with the ancient DNA. Again, except in the case of high concentration of extracts, no sign of contamination was detected in either the extraction negative controls or the amplification negative controls. In some cases, primer-dimer, an amplified product of 40 to 80 nucleotide bases consisting of two to four repeats of the approximately 20 base primer used in the reaction, was detected (see figure 1). The ability of the DNA polymerase to amplify the primer-dimer in the presence of the DNA extracts is typical of reactions lacking template DNA, and is inconsistent with the presence of PCR inhibitors. If PCR inhibitors were present, the Taq enzyme would be of highly reduced activity, and would not catalyze the amplification of primer-dimer. The presence of primer-dimer therefore attests to the normal functional state of the Taq enzyme. In the case of amplifications using high concentrations of extract, weak amplification was detected in some of the sample extracts and also in some of the extraction negative controls. Six amplifications, using the primers specific to the A, B, C, D and X fragments, were performed using high concentrations of extract. In the case of the C fragment (74 bases long) no amplification was detected. In the case of the B, D and two X fragments (122 bases, 111 bases, 162 and 194 bases long respectively) several of the bone sample extracts amplified weakly, as did several of the extraction negative controls (see figure 2). In the case of the A amplification (175 bases long) every extract amplified weakly, clearly the result of contamination. Which samples and negative controls amplified and which did not were not consistent between amplifications. Upon overnight digestion with the appropriate restriction enzyme, it was found that the results from the sample extracts were identical to those of the negative controls that amplified (and identical to those found in FAK, a member of haplogroup I).

Table 3: Kennewick Amplification and Restriction Digest Results

Extraction:	Sample	Amplifications Hap A	Hap B	Hap C	Hap D	A conc.[1]	B conc.	C conc.	D conc.	X1715 conc.	X 14465 conc.
Silica	Ken. Rib	neg[2]	neg	neg	neg	weak pos[3]	weak pos (-)[5]	neg	neg	neg	neg
	Ken. M-carpal	neg	neg	neg	neg	weak pos	pos (-)	neg	weak pos (?)	neg	neg
	EDTA neg. 1	neg	neg	neg	neg	weak pos	pos (-)	neg	weak pos (?)	neg	pos (-)
	EDTA neg. 2	neg	neg	neg	neg	weak pos	neg	neg	neg	neg	neg
	EDTA neg. 3	neg	neg	neg	neg	weak pos	weak pos (-)	neg	neg	neg	pos (-)
	Pro-K neg.	neg	neg	neg	neg	weak pos	neg	neg	pos (+)	neg	neg
	Rib 1:10 dil.	-	neg	neg	neg	-	-	-	-	-	-
	Rib 1:50 dil.	-	neg	neg	neg	-	-	-	-	-	-
	M-carpal 1:10	-	neg	neg	neg	-	-	-	-	-	-
	M-carpal 1:50	-	neg	neg	neg	-	-	-	-	-	-
	Rib pro-K	-	neg	-	neg	-	-	-	-	-	-
	M-carpal p-K	-	neg	-	neg	-	-	-	-	-	-
	pro-K neg.	-	neg	-	neg	-	-	-	-	-	-
Phenol-Chlor.	Ken. Rib	-	neg	-	neg	weak pos	pos (-)	neg	neg	neg	neg
	Ken. M-carpal	-	neg	-	neg	weak pos	pos (-)	neg	neg	neg	pos (-)
	EDTA neg. 1	-	neg	-	neg	weak pos	neg	neg	neg	neg	neg
	EDTA neg. 2	-	neg	-	neg	pos	neg	neg	neg	neg	neg
	EDTA neg. 3	-	neg	-	neg	weak pos	pos (-)	neg	neg	neg	neg
	Pro-K neg.	-	neg	-	neg	weak pos	neg	neg	neg	neg	neg
	Rib 1:10 dil.	-	neg	-	neg	-	-	-	-	-	-
	Rib 1:50 dil.	-	neg	-	neg	-	-	-	-	-	-
	M-carpal 1:10	-	neg	-	neg	-	-	-	-	-	-
	M-carpal 1:50	-	neg	-	neg	-	-	-	-	-	-
Gene-Releaser	Ken. Rib	-	neg	-	-	-	-	-	-	-	-
	Ken. M-carpal	-	neg	-	-	-	-	-	-	-	-
	EDTA neg. 1	-	neg	-	-	-	-	-	-	-	-
	EDTA neg. 2	-	neg	-	-	-	-	-	-	-	-
	EDTA neg. 3	-	neg	-	-	-	-	-	-	-	-
	Pro-K neg.	-	neg	-	-	-	-	-	-	-	-
Silica w/o EDTA	Ken. Rib	-	neg	-	neg	weak pos	neg	neg	weak pos(+)	pos (-)	-
	Ken. M-carpal	-	neg	-	neg	pos	weak pos (-)	neg	pos (+)	pos (-)	-
	Pro-K neg.	-	neg	-	neg	weak pos	neg	neg	pos (?)	pos (-)	-
	Rib 1:10 dil.	-	neg	-	neg	-	-	-	-	-	-
	Rib 1:50 dil.	-	neg	-	neg	-	-	-	-	-	-
	M-carpal 1:10	-	neg	-	neg	-	-	-	-	-	-
	M-carpal 1:50	-	neg	-	neg	-	-	-	-	-	-

1 Conc. = using high concentration of extract

2 Neg = no amplification detected

3 Pos = amplification detected

4 - = no amplification attempted

5 Within parentheses, a '-' indicates no digestion, a '+' indicates positive digestion, and a '?' indicates such a signal too weak to determine results

Thus, the results from the positive amplifications from both the Kennewick samples and negative controls are not consistent with a source that is a member of haplogroups A, B, C, D or X, but are consistent with a source that is a member of haplogroup I (such as FAK). Because ancient DNA is highly damaged, amplification success is generally correlated with the length of the amplified product (shorter fragments being easier to amplify). Note that there is no correlation between amplification success and fragment length in this case, suggesting that the amplified DNA is not ancient. These results are consistent with a very low level of contamination of the extracts with modern DNA, most likely from FAK, but possibly originating at the reagent or lab disposables manufacturer. Although disposable pipette tips and tubes utilized during the extraction are certified DNA-free by the manufacturer, in reality the manufacturer's quality control methods will only detect DNA presence above 5×10^{-11} mg (Eppendorf™). In addition, although all reagents utilized are produced in extremely sterile conditions and additionally filtered and/or Ultra-Violet irradiated in the laboratory, low levels of DNA contamination might still be present. However, haplogroup I, defined by the loss of the DdeI restriction site at nucleotide position 1715 and distinguished from haplogroup X by the absence of the AccI restriction site at nucleotide position 14465, is very rare among Europeans (generally 5% or less in frequency), and absent in all other populations except for extremely rare examples in the middle east (Brown et al, 1998; Torroni et al., 1994,1996). Therefore the most likely source of this DNA is FAK.

Because any contamination is extremely minor it is only detectable when large concentrations of DNA extract are utilized in amplifications, and even then it is only detectable in a minority of cases. The amplification process will only amplify the target fragment if at least one copy of that fragment is present in the DNA extract that is added to the reaction, and in fact is very inefficient at such low concentrations of DNA. Thus, if the DNA in the extract is at such low concentrations that the probability of obtaining even one copy in the amount added to the PCR reaction is quite low, the result will be only intermittent amplification. For example, if there are only five copies of the target DNA in the total of 50 microliters of extract, there is only a 10% chance that one of these copies will be contained in any one microliter portion of the extract. However, much larger amounts of the extract are used in the amplification, as in the high concentration amplifications above, the probability that at least one copy of your target DNA will be present is much higher. Thus very low levels of contamination are likely to be detected only when high concentrations of DNA extract is used in the amplification process. Given that it is almost impossible to prevent contamination at these extremely low levels, most ancient DNA amplifications using high concentrations of extract will eventually detect contamination.

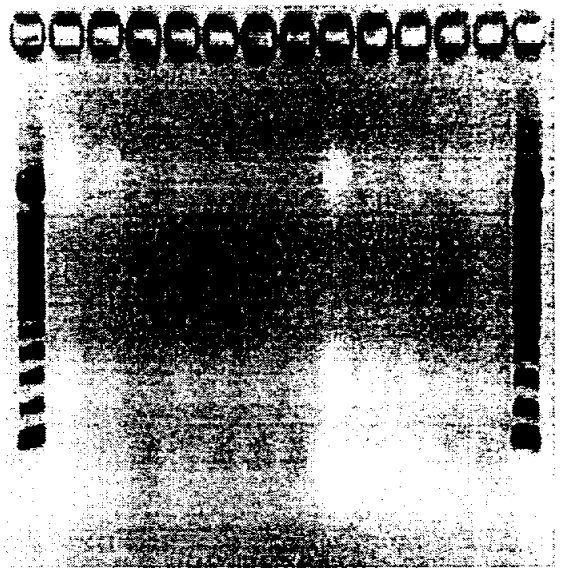

FIGURE 1: Gel image showing haplogroup B amplification results from Kennewick and negative control silica extracts. Lanes one and 14 contain 100 bp size marker (bands increase in size by 100 nucleotide bases, lowest band equals 100 bases). Lane 2: Kennewick Rib, Lane 3: Kennewick Metacarpal, Lane 4: Kennewick rib 1:10 dilution, Lane 5: Kennewick Metacarpal 1:10 dilution, Lane 6: Kennewick Rib 1:50 dilution, Lane 7: Kennewick Metacarpal 1:50 dilution, Lanes 8-10: EDTA negative controls, Lane 11: proteinase K negative control, Lane 12: chimp control, Lane 13: negative amplification control. No amplifications seen (amplified band should be 122 bases, between two lowest size marker bands). Primer-dimer observed as dark smudges in majority of lanes, below the level of the lowest size marker band.

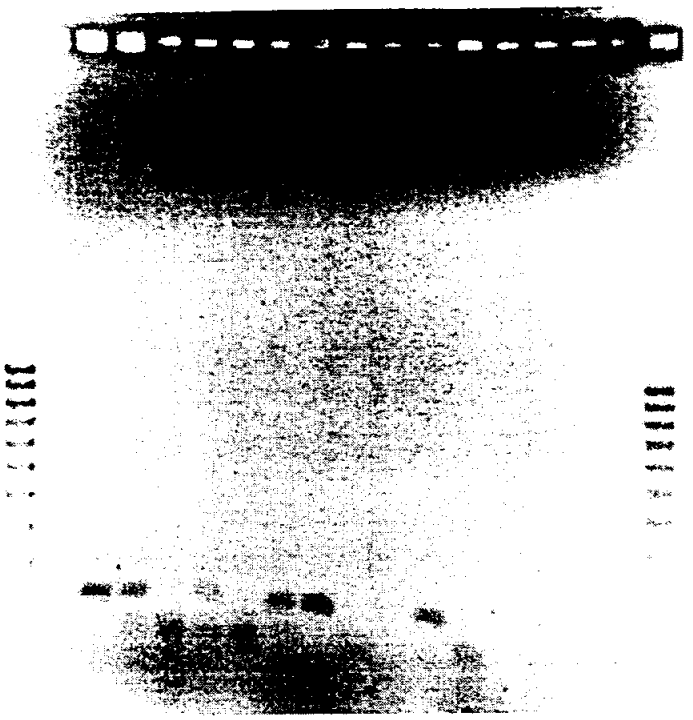

FIGURE 2:Gel image showing haplogroup B amplifications utilizing high concentrations of extracts. Lanes 1 and 18: 100 base-pair size marker, Lane 2: Silica-extracted rib, Lane 3: Silica-extracted metacarpal, Lanes 4-6: Silica-extracted EDTA negative controls, Lane 7: Silica-extracted proteinase K negative control, Lane 8: Phenol/chloroform extracted rib, Lane 9: Phenol/chloroform extracted metacarpal, Lanes 10-12 Phenol/Chloroform extracted EDTA negative controls, Lane 13: Phenol/chloroform extracted proteinase K negative control, Lane 14: Non-decalcified silica-extracted rib, Lane 15: Non-decalcified silica-extracted metacarpal, Lane 16: Non-decalcified proteinase-K negative control, Lane 17: amplification negative control. Note presence of appropriate sized bands in lanes 2, 3, 4, 6, 8, 9, 12, and 15, and primer-dimer sized bands in many of the lanes.

In conclusion, the inability to amplify DNA except at extremely high extract concentrations (and then with corresponding sporadic amplification of negative controls), the lack of evidence for PCR inhibition, the inconsistencies in which extracts amplified at high concentration, the identical restriction digest results for Kennewick samples and negative extract controls to those of FAK, and the lack of correlation between amplification success and fragment length are most consistent with the absence or very low concentration of ancient DNA in the two bone fragments under analysis and the presence of very low levels of modern contamination in these extracts. Thus available technology and protocols do not allow the analysis of ancient DNA from these remains. However, it is important to note that the success in extraction and amplification of ancient DNA does not seem highly dependent on the age of the sample, and several humans of similar or significantly older age have successfully been analyzed, both from the Americas (e.g., Kaestle 1997, 1998; Stone and Stoneking 1996) and from elsewhere (e.g., Krings et al. 1997; Naumova et al. 1998). It must be emphasized that the lack of

success in amplifying ancient DNA from one sample has little bearing on the probability of success in the analysis of another.

DOI 09861

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APPENDIX A

Ancient DNA Extraction Protocols:

1. Use molecular or HPLC grade water that has been checked for contamination by PCR amplification using human primers.
2. Use unopened or ancient DNA designated chemicals, and molecular-grade and/or DNAse/RNAse/DNA free reagents. (e.g., Tris, EDTA, NAOH, TE).
3. Use glassware and/or plasticware that was treated for use with ancient materials (UV irradiated, bleach or HCl decontaminated, guaranteed DNA free when possible).
4. When appropriate, solutions should be filtered through a 0.22 μm filter to remove any contaminating DNA.
5. All solutions should be pHed with a designated ancient DNA pH meter or by using sterile filter-barrier pipette tips with pH strips.
6. Extractions should be performed in a designated ancient DNA laboratory, which is isolated from PCR amplification products and bleach and/or UV sterilized routinely.
7. Throughout procedures, aDNA lab coats, latex gloves and facemasks should be worn.

Decontamination of bone surfaces and powdering of sample:

1. Bone surfaces are cleaned with 10% bleach using either kimwipes or a bleach-sterilized toothbrush, to destroy DNA surface contaminants. In some cases the bone may be soaked in 10 bleach for 5 minutes instead.
2. Bone surfaces are cleaned with UV-sterilized molecular grade water.
3. OPTIONAL: bone surfaces are cleaned with UV-sterilized ethanol
4. Each bone surface is UV irradiated at 254 nm for at least 5 minutes at less than 5 inches from UV source to destroy any remaining DNA surface contaminants.
5. Bone is cracked open to expose internal surfaces (either by hand or using an awl and hammer or dremel tool with cutting disk). If this is not possible, bone surfaces are scraped away using a bleached and UV-irradiated razor blade or scalpel.
6. Internal surfaces are removed in powdered form using a dremel tool with an engraving cutter bit.
7. Bone powder is stored in a UV-irradiated sterile 2ml to 50ml polypropylene tube.

Decalcification of bone:

1. 0.15 to 2 gm of bone powder is transferred to a UV-irradiated sterile 2 ml or 15 ml polypropylene tube.
2. 1.5 to 4 ml UV-irradiated sterile 0.5 M EDTA (pH 8.0) is added to each sample and to an empty 2 ml or 15 ml UV-irradiated sterile tube (as a negative control).
3. Tops of tubes are parafilmed.
4. Samples are decalcified at 4°C for 12 - 24 hours with mild rocking or rotation.
5. Samples are centrifuged and EDTA is removed.

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6. 1.5 to 4 ml UV irradiated 0.5 M EDTA (pH 8.0) is added to each sample, the first negative control, and an additional negative control.
7. Tops of tubes are parafilmed.
8. Samples are decalcified at 4°C for 12 - 24 hours with mild rocking or rotation.
9. Samples are centrifuged and EDTA is removed.
10. EDTA decalcification (steps 6-9) is repeated at extractor's discretion.

NOTE: steps 11-15 are unnecessary if proteinase K digestion will be carried out using an EDTA proteinase K buffer.

11. 1.5 to 4 ml UV-irradiated molecular grade water is added to each sample, each negative control, and an additional negative control.
12. Tops of tubes are parafilmed.
13. Samples are rocked or rotated for 10 minutes.
14. Samples are centrifuged and water is removed.
15. Water wash (steps 11-14) is repeated at extractor's discretion.

Proteinase K Digestion:

1. 1 to 2 ml of UV-irradiated proteinase K buffer is added to each sample and negative control, plus an additional negative control. Proteinase K buffer can be one of three types:

1. ATL buffer, purchased from Qiagen™
2. EDTA buffer: 0.5 M EDTA pH 8.0
   0.5% SDS
3. Non-EDTA buffer: 50mM (.05M) Tris pH 8.0
   1mM $CaCl_2$
   1mM DTT
   0.5 % Tween 20

2. Proteinase K enzyme is added to each sample and the negative controls for a final concentration of 1 mg/ml.
3. Tops of tubes are parafilmed.
4. Samples are digested overnight at 37°C or 55°C with mild rocking or rotation.

DNA Extraction: Many options exist, including Phenol Chloroform or Silica
Phenol Chloroform extractions have the advantage of providing a larger quantity of DNA while Silica extractions have the advantage of eliminating more of the amplification inhibitors that commonly co-extract with DNA. Recently, another option, the BioVentures GeneReleaser kit, has become available, which involves a proprietary reagent that "sequesters cell lysis products which might inhibit polymerases" and "avoids lengthy protocols and excessive sample manipulations which may introduce contamination", (BioVentures, Inc., Murfreesboro, TN, product literature, 1996). This protocol prepares only enough extract for one amplification.

Phenol Chloroform Extractions:

1. An equal volume of molecular grade phenol, pH 8.0, is added to the proteinase-K digested samples and negative controls.
2. Tops of tubes are parafilmed.
3. Samples are rocked or rotated at room temperature for 10 minutes.
4. Samples are centrifuged at high speeds to separate layers.
5. Supernatant containing DNA (note that the DNA layer is frequently the bottom layer if the EDTA buffer is used during proteinase K digestion) is removed and transferred to a new UV-irradiated tube. Tube containing phenol and any remnants of bone powder is retained for return to DOI.
6. An equal volume of molecular grade phenol, pH 8.0, or phenol:chloroform:isoamyl alcohol (25:24:1) is added to the supernatant.
7. Tops of tubes are parafilmed.
8. Samples are rocked or rotated at room temperature for 10 minutes.
9. Samples are centrifuged at high speed to separate layers.
10. Supernatant containing DNA is removed and transferred to a new UV-irradiated tube.
11. An equal volume of molecular grade chloroform:isoamyl alcohol (24:1) is added to the supernatant.
12. Tops of tubes are parafilmed.
13. Samples are rocked or rotated at room temperature for 10 minutes.
14. Samples are centrifuged at high speeds to separate layers.
15. Supernatant containing DNA is removed and transferred to a new UV-irradiated tube.
16. Samples are centrifuged at high speed to separate supernatant from any remaining chloroform.
17. Supernatant containing DNA is removed and transferred to a Centricon-100 or Centricon-30 centrifugal filtration unit (Millipore™).
18. Samples are concentrated and filtered through centrifugation in a Centricon unit using manufacturer's protocol, to dryness.
19. 500 to 1000 ul UV-irradiated molecular grade water is added to each Centricon unit to wash samples, and unit is centrifuged per manufacturer's protocol, to dryness.
20. 500 to 1000 ul UV-irradiated molecular grade water is added to each Centricon unit to wash samples, and unit is centrifuged per manufacturer's protocol until sample volume of 50 to 150 ul is reached.
21. Samples are retained in UV-sterilized final collection tubes per manufacturer's protocol.
22. Samples are transferred to a UV-irradiated 0.5 ml or 2 ml sterile tube and frozen.

Silica Extractions (modification of Yang et al. 1998 AJPA 105(4):539-544):

1. The Proteinase K digested samples and negative controls are centrifuged at high speed.
2. The supernatant is removed to a UV-irradiated 2 ml or 15 ml polypropylene tube. Original proteinase K digestion tube with any remaining bone powder is retained for return to DOI.

3. Samples are concentrated in a Centricon-100 or Centricon-30 concentration unit per manufacturer's protocol, to sample volume of approximately 30 ul, and samples are transferred to UV-irradiated collection tubes.
4. 5 volumes of Qiagen PB Buffer from the QiaQuick PCR purification kit (Qiagen™) is added to each Centricon collection tube and mixed with sample.
5. Samples are loaded 750 ul at a time onto the Qiagen column.
6. Columns are centrifuged at 12,800 x g for 1 minute. Columns are reloaded and centrifuged until all sample is exhausted.
7. Samples are washed with 750 ul of Qiagen PE buffer from the QiaQuick PCR purification kit and centrifuged for 1 minute per manufacturer's protocol.
8. Qiagen columns are placed into final irradiated collection tubes.
9. DNA is eluted from columns by loading 100 ul of Qiagen EB buffer (or UV-irradiated TE or molecular grade water), incubated at room temperature for 5 minutes, and columns are centrifuged for 1 minute per manufacturer's protocol.
10. Samples are frozen.

Note: On occasion, DNA extracts from the Phenol Chloroform protocol are later run through the Silica extraction protocol to remove co-extracted PCR inhibitors.

GeneReleaser Extractions

1. The Proteinase K digested samples and negative controls are centrifuged at high speed.
2. The supernatant is removed to a Centricon-100 or Centricon-30 unit. Original proteinase K digestion tube with any remaining bone powder is retained for return to DOI.
3. Samples are concentrated in a Centricon-100 or Centricon-30 concentration unit per manufacturer's protocol, to sample volume of approximately 30 ul, and samples are transferred to UV-irradiated collection tubes.
4. 3 ul of each sample and negative control extract are transferred to DNA-free 200 ul PCR tubes.
5. 10 ul of vortexed GeneReleaser is added to each PCR tube, and tubes are vortexed to mix.
6. Tubes are microwaved on high for 6 minutes.
7. Tubes are incubated in an 80°C water bath for 10 minutes.
8. PCR reactions are carried out directly in the PCR tubes in which extraction took place, compensating for the increased volume of extract by reducing the amount of water added.

PCR Amplification:

Amplifications are set up in the pre-PCR ancient DNA laboratory. A PCR cocktail is prepared that contains all reagents [0.6 mM primers, 1X manufacturer's buffer with 1.5 mM $MgCl_2$, ddH_2O, 1 mg/ml BSA, 200 nM dNTPs] except the Taq polymerase

and the ancient DNA template was UV cross-linked for 10 minutes to eliminate any exogenous DNA contamination. Standard ancient DNA primers for the regions encompassing the haplogroup-defining mutations and the hypervariable segments are employed (for example, see Stone and Stoneking (1993) American Journal of Physical Anthropology 92:463-471, Parr, Carlyle and O'Rourke (1996) American Journal of Physical Anthropology 99:507-518). The equivalent of "hot start" amplifications are performed in 25 - 50 µl volumes using 3-10 µl ancient DNA extract and 1.5 - 3 units of Amplitaq Gold DNA polymerase (Perkin Elmer) or Platinum Taq DNA polymerase (Gibco BRL). Tubes are sealed and transported to the physically separate PCR laboratory. The amplifications are subjected to an initial 2-11 minute denaturation step at 95°C, then 35 to 45 cycles in which reactions are denatured at 95°C for 30-15 seconds, annealed at 49-59°C for 30-15 seconds, and extended at 72°C for 25-60 seconds, followed by a final extension step of 3-5 minutes at 72°C. At least one negative control, consisting of PCR cocktail, Taq DNA polymerase, and 3-5 ul sterile water, is included in each amplification set. In some cases the DNAse predigestion protocol (Eshleman and Smith 2000) was utilized. In this protocol, the PCR cocktail is prepared without the primers but including the Taq. Amplification grade DNAse is added to the cocktail to digest any contaminating DNA, and after incubation at room temperature for 10 minutes the DNAse is heat inactivated at 70°C. The primers are added and the cocktail is aliquoted into the PCR tubes. PCR then proceeds as usual.

Haplogroup Assignment

Five to ten µl of amplification product containing the restriction site gains or losses that characterizes haplogroups A, C, D and X are digested overnight at 37°C with 5 units HaeIII, HindII/AluI, AluI and DdeI/AccI respectively, according to manufacturers' recommendations. Restriction fragments and the presence/absence of the 9 bp deletion are resolved on 6% polyacrylamide gels or 2-3% agarose gels, stained with ethidium bromide and visualized, then photographed, on a UV transilluminator using a digital imaging system.

Sequence Reactions:

The remaining PCR product (approximately 45 µl) from successful amplifications of D-loop fragments is purified, per manufacturer's protocol, using either a Microcon-30 or Microcon-100 microconcentrator, to a volume of 30 µl. Sequence reactions are performed according to manufacturers' recommendations (although in some cases reduced to a 10 µl volume), using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS, Big Dye Terminator chemistry (Perkin Elmer/ABI). Samples are purified using ethanol precipitation (95% ethanol, 3M sodium acetate) according to manufacturers' protocols. Samples are rehydrated with 3 µl of sequencer loading dye and heated at 90°C for 2 minutes. Reactions are run on an ABI PRISM 377 DNA Sequencer using a 4.25% Acryl/Bisacrylamide or 5% LongRanger gel (Perkin Elmer/ABI) and data is analyzed using ABI Prism™ Sequencing software. Sequences are aligned by hand to the Cambridge reference sequence (Anderson et al. (1981) Nature 190:457-465). Both directions for each fragment are sequenced.

Cloning:

In some cases of suspected contamination PCR products are cloned and individual clonal colonies are sequenced to detect any variant sequences. In this case, PCR products are cloned using the Topo TA Cloning Kit (Invitrogen™), using the manufacturer's recommended protocol. 4 ul of the PCR product is utilized in the cloning procedure, and a minimum of 5 clonal colonies are grown overnight in LB broth. The Qiagen™ mini prep kit is then used to prepare clones for sequencing, according to manufacturer's recommended protocol.