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Date: 9/20/00 4:15 PM
Priority: Normal
TO: Jason Roberts at NP-WASO-DCA
Subject: Final Report on Analysis of Kennewick Remains

Jason

Find attached our final report of our analysis of the Kennewick remains. Please let me know if you have additional recommendations for changes.

david

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

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U.C. Davis Molecular Anthropology Laboratory

The Molecular Anthropology Laboratory in the Anthropology Department at U. C. Davis consists of three separate rooms in which separate tasks are performed: 1) the ancient DNA extraction/prep lab (approximately 300 square feet), 2) the modern DNA extraction/prep lab (approximately 500 square feet) and 3) the PCR (polymerase chain reaction) amplification and analysis lab (approximately 750 square feet). The ancient DNA extraction/prep laboratory is equipped with two half-inch, UV irradiated plexiglass glove boxes each with dedicated supplies and equipment. These glove boxes are bleeched and decontaminated monthly. The air in the ancient DNA extraction/prep laboratory is subjected to extra filtration and designed (e.g., all external portals are sealed) to maintain positive pressure at all times. Access to the ancient DNA lab is strictly limited and all labcoats, gloves, masks and haircovers used here are dedicated for use only in this lab. Double-gloved entry is made routinely via the portals to each glove box to prevent carry-over contamination from outside the lab and all PCR preps are prepared in borosillicate microcapillary tubes and flame sealed inside the glove box so that PCR reactions cannot be contaminated during or after transporting them to the PCR amplification and analysis laboratory.

The PCR amplification and analysis lab is equipped with five thermal cyclers, including three Rapid Air Cyclers (Idaho Technology),which are used predominantly for amplification of ancient DNA, an ISO 2000 imaging system and an ABI 310 Genetic Analyzer (DNA sequencer). Other equipment in this lab that are employed in the analysis of ancient DNA include a vacuum dryer, UV crosslinker, UV transilluminator, various power supplies and electrophoresis units, refrigerators and freezers, two MacIntosh G4 computors and one MacIntosh G3 computor that interfact this equipment and are used for data analysis. All three labs are equipped with standard lab equipment such as freezers, refrigerators, pH meters, electronic scales, centrifuges, pipettemen,etc., and utilities (water,air and vacuum).

Historical Context

On August 9, 1996 one of my graduate students (now, Dr.) Frederika A. Kaestle (FAK), was contacted by Dr. James Chatters of Applied Paleoscience in Richland, Washington and asked to conduct a DNA analysis of recently recovered skeletal remains. These remains had been discovered on July 28, 1996 eroding from a bank of the Columbia River at Columbia Park in Kennewick, Washington. Because the remains exhibited "caucasoid-like" morphometric traits, yet harbored a projectile point (later determined to be of the Cascade variety, typically manufactured as early as 7,000 years B.P.), embedded in its right ilium, Dr. Chatters asked if we were able, and willing, to perform the DNA analysis required to determine whether or not the sample exhibited DNA characteristics typical of American Indians.

On August 12, after concluding that such an analysis was consistent with the research objectives of the U. C. Davis Molecular Anthropology Laboratory, of which I am Director, and should be conducted pro bono, I advised FAK to inform Dr. Chatters that we would do this analysis. On August 19th the Coroner of Benton County, Washington, authorized Dr. Chatters to request that the remainder of a bone sample that had been sent to Dr. Erving Taylor's lab at U.C. Riverside for radiocarbon dating be forwarded on to my lab for DNA analysis after completion of the radiocarbon dating. After completion (on August 26th) of the radiocarbon dating analysis (which led to the estimate that the remains were about 8,400 years old), an approximately 1.4 gram portion of remaining bone was sent (on September 5) to FAK by Dr. Donna Kirner, manager of Dr. Taylor's radiocarbon dating laboratory.

Preliminary Analysis

On October 1 FAK began her study of the Kennewick bone specimen (WA-1) attempting her first (of two) consecutive DNA extractions (one each on October 6 and October 22) from an approximately 0.25 gram portion of the sample. After the surfaces of the sample were UV irradiated to crosslink possible DNA contaminants on its surface, the sample was ground to powder, then thrice decalcified with EDTA at 4°C. The EDTA solution was poured off, then washed thrice with UV irradiated ddH_20. Proteinase K and buffer were added to the tube and the sample was digested (i.e. deproteinized) overnight on a rocker at 55°C. DNA was extracted successively with phenol, phenol/chloroform, then chloroform, filtered in a Centricon-100 unit and washed twice with ddH_20.

The appropriate PCR primers (see Table 1) were used to amplify fragments containing the restriction sites diagnostic of mitochondrial DNA (mtDNA) haplogroup A and haplogroup D and the 9 bp deletion that is characteristic of haplogroup B (see Table 1). While the fragment amplified using primers for the 9 bp deletion lacked the deletion, the negative amplification controls were also positive, indicating possible contamination of the sample with modern DNA (it was concluded that the haplogroup B PCR primers were contaminated, since all reagents except the haplogroup B primers had also been used to amplify the PCR fragments containing the restriction sites characteristic of haplogroups A and D).

The fragments containing the restriction sites (the gain of a Hae III site at np 663 and the loss of an Alu I site at np 5176) characteristic of haplogroups A and D, respectively, were digested overnight in a 37°C H_20 bath with the appropriate restriction enzymes. While the A fragment of the Kennewick sample was not digested by Hae III, indicating absence of the restriction site characteristic of haplogroup A, the D fragment exhibited only slight digestion (a lack of digestion indicates membership in haplogroup D). Since the positive control itself was not fully digested, low enzyme activity could be responsible for this slight "haplogroup D signal." The D fragment from this same extraction was then re-amplified and restricted again with Alu I and, this time, exhibited no digestion at all, as shown in figure 1a (the amplification positive digested significantly toward completion), suggesting that the sample might be a member of haplogroup D. Note, in figure 1a, that one of the EDTA negative controls also amplified but completely digested, thus the same (modern?) source could not have contaminated the Kennewick

mtDNA (which did not digest at all). Moreover, subsequent amplifications of both the A and the D fragments yielded contamination in some negative controls that also digested to completion (and therefore the source of this contamination is not a member of haplogroup D). Since the control samples that were contaminated differed between the A and D fragment amplifications, contaminated tubes are likely to be responsible.

To try to replicate the absence of an Alu I restriction site at np 5176 (characteristic of haplogroup D) in the Kennewick sample, FAK conducted a second extraction on another 0.25 gram portion of the Kennewick remains that had been ground to powder on October 1, following the same procedure as before. While the A fragment failed to amplify (a simultaneous digestion of the A fragment from the first extraction did, in fact, amplify again) the D fragment did amplify but this time was almost completely digested by the Alu I restriction enzyme (figure 1b), conflicting with the result of the digestion of the D fragment amplified from the first extraction. Both the EDTA and PK negative controls also amplified (and were either completely or partially digested by Alu I). A second amplification of the D fragment from this second extraction also digested to completion. Two additional amplifications of the D fragment were next conducted from the first extraction; one of these digested partially while the other digested completely. In figure 1c, these two digestions are shown together with that of the second amplification of the second extraction.

Thus, two separate amplifications from two different extractions suggested that Kennewick Man does not belong to haplogroup D (because the fragment was at least partially digested at np 5176 by Alu I restriction enzyme) while a single amplification from one of the two extractions suggests he might belong to haplogroup D. Given that at this point it was still unclear whether or not either of the two extractions were clean (i.e., uncontaminated), amplifications from the extracts had given conflicting information and neither extract had been successfully tested for the diagnostic markers for haplogroups B, C or X, it was impossible to determine to which, if any, of the common modern American Indian mtDNA haplogroups the Kennewick remains belong. At very most, our results provided, at this point, no evidence that the Kennewick remains belonged to haplogroups A, B or C.

Premature termination of the study

On, or about, October 19th, I received a letter from Walla Walla Army Corps of Engineers (COE) Counsel Linda Kurts demanding that we discontinue DNA analysis of the Kennewick remains. At her request, I called Dr. Ray Tracy, archaeologist for the COE, for clarification and told him I would discontinue this analysis I had promised to conduct only after assessing the propriety of such action. After conferring with, and on the advice of, Steven Drown, the University of California at Davis Campus Counsel, I contacted Mr. Floyd Johnson, Coroner of Benton County, Washington, who had authorized DNA testing of the sample to confirm that he now wished us to discontinue our DNA analysis. He verbally confirmed this and, at my request, sent me a written record of this confirmation on October 29th. Between October 27th, 1996 and early May of 1999, no analyses of the Kennewick remains were conducted in my laboratory. During

this period of time the Kennewick remains were locked in a fire-proof safe, whose combination was known only to me, in my office.

Again, upon advice of my Campus Counsel, Steven Drown, I agreed to surrender the sample of the Kennewick remains in my possession, but refused to return all documents, notes, photographs, etc. that FAK had generated between October 1st and October 28th. A subsequent plan to retrieve the Kennewick sample from my possession was aborted for reasons of which I have no direct knowledge, and the sample remained in the safe in my office until early February of 1999. On February 2, 1999, I surrendered the remaining approximately 0.9 gram portion of the Kennewick sample in our possession to Mr. Tim Simmons, an attorney for the Justice Department, who transported the specimen, packaged to my specifications, to the Burke Museum on the campus of the University of Washington in Seattle where, in the interim, the Kennewick remains had been transferred.

Recovery of Sample for DNA Analysis

During the early spring of 2000, the Department of the Interior decided to proceed with DNA study of the Kennewick remains in order to better inform their disposition determination. On April 24th, I visited the Burke Museum to participate in the selection of samples of the Kennewick remains for DNA analysis by three different laboratories (including my own). I had previously expressed my own desire to resume custody of the 0.9 gram sample that I had surrendered on February 2, 1999, and complete the DNA analysis that FAK had initiated. After confirming that the sample of the Kennewick remains that I surrendered to attorney Tim Simmons on February 2, 1999, remained as FAK and I had originally packaged it for transfer to (and storage with the other Kennewick remains housed at) the Burke Museum, I accepted the return of custody of these remains from Dr. Michael (Sonny) Trimble, Director of the St. Louis District COE Mandatory Center of Expertise for the Curation and Management of Archaeological Collections (MCX-CMAC) and returned (on April 26th) to my laboratory at U.C. Davis to complete the DNA analysis initiated in my lab by FAK.

Upon my return, I gave approximately equal portions of the remaining 0.9 gram sample to each of my two senior graduate students, Mr. Ripan S. Malhi (RSM) and Mr. Jason A. Eshleman (JAE), who had agreed to complete the analysis begun by FAK. The evidence I gathered during my review of documents at the Burke Museum pertaining to the history of discovery and study of the Kennewick remains, and which were described in my earlier report to Dr. Francis P. McManamon of the National Park Service, leads me to conclude that the single element least exposed to modern contamination after the remains were discovered is the fifth metacarpal element submitted by Dr. Chatters to Dr. Taylor's lab at U.C. Riverside for radio-carbon dating on August 5, 1996, prior to the time most elements were handled by either known or unknown individuals. This was in fact the sample of the Kennewick remains previously studied by FAK in my lab and with which I returned to my lab on April 26, 1999. That particular element was reported to have been recovered in a matrix of hardened clay inside the cranium of the Kennewick remains and is known to have been handled only by a few individuals from each of whom we obtained a sample of DNA. DNA from these few individuals could then be compared

to DNA suspected of being a modern DNA contaminant co-extracted with the intrinsic ancient DNA extracted from the Kennewick remains.

Resumption of DNA Study of the Kennewick Remains

Samples were given, in flame sealed tubes, by David Glenn Smith (DGS) to RSM and JAE. All reagents were tested for the presence of contaminants prior to extractions by RSM and JAE by attempting to amplify human mtDNA (bp 16209 to bp 16356) using the reagents as a reaction template for PCR. No reagents showed evidence of contamination. The PCR primers employed for all amplifications and restriction enzymes that identify each haplogroup tested are given in Table 1 below.

Extractions of DNA

RSM

The bone sample was UV irradiated on each side for 10 minutes to eliminate surface contamination. An extraction was performed using approximately 0.46 grams of (uncrushed) bone. The remains were transferred to a 15 ml falcon tube and immersed in 0.5 ml of sterile filtered 0.5 M EDTA. A control blank (XN-1) was also immersed in 0.5 M EDTA and both were placed on a rocker at 4°C for 24 hours. The tubes were spun in a centrifuge and the EDTA was removed and stored in a sterile 50ml tube.

1.5ml Qiagen ATL digestion buffer was added to the bone remains. Qiagen proteinase K (pK) was added to the buffer to a final concentration of 1 mg/ml. The samples were sealed and incubated overnight at 55°C. DNA was extracted from the pK digest using the phenol/chloroform method. An equal volume of phenol was added to the pK digest and vortexed. The mixture was then centrifuged and the aqueous layer was removed. An equal volume of phenol/chloroform was added to the sample, vortexed, centrifuged, and the aqueous layer was removed. Finally, an equal volume of chloroform was added to the sample, vortexed, centrifuged, and the aqueous layer was removed. The same process was completed for the XN-1 control blank. The DNA extract and XN-1 control blank were frozen overnight in the -20°C freezer.

No amplifications were obtained using the phenol/chloroform (p/c) DNA extract and all control blanks and negative controls were clean. The p/c DNA extract was then purified and concentrated using the silica spin column based DNA extraction protocol by Yang et al. (1998) and stored at -20°C. Attempts were made to amplify regions containing diagnostic restriction sites for haplogroups A, C, D and X, the 9bp deletion (Haplogroup B) and 2 segments of HVI (bp15938 to bp16139 and bp16209 to bp16356) using the PCR primers given in Table 1 and to digest the PCR products with restriction enzymes that recognize restriction sites diagnostic for each of these haplogroups (also given in Table 1).

JAE

Parallel extractions were performed on a 0.29 gram fragment (WAJA) and a 0.15 gram fragment (WAJB) sealed in two different flame-sealed 1.5 ml eppendorf tubes provided to JAE by DGS. Both fragments were washed with a 10% bleach solution for 3 minutes to eliminate surface contamination then washed with sterile ddH2O to remove bleach. Bone (uncrushed, to minimize the opportunity for contamination by modern DNA during analysis) was then UV irradiated for 10 minutes per side to further eliminate surface contamination. The remains were transferred to sterile 2.0ml tubes and immersed in 1.5ml sterile filtered EDTA. Extractions of the contents of a separate tube were performed in a manner identical to that employed for the sample extractions, except no sample was placed in the tube (extraction control). All tubes were sealed with parafilm and placed on a rocker at 4°C for 24 hours. The tubes were spun in a centrifuge and the EDTA was removed and stored in a sterile 2.0ml tube.

1.5ml Qiagen ATL digestion buffer was added to each tube. Qiagen proteinase K was added to the buffer to a final concentration of 1mg/ml. The samples were sealed with parafilm and incubated at 55°C overnight. Supernatant from digested samples were concentrated using Amicon YM-30 concentrators to a volume of 100ul. Extractions on both samples and the extraction control were performed using silica spin columns following a modified version of the protocol described by Yang et al. (1998). Elutions of the sample and the extraction control were stored overnight at -20°C.

1:5 and 1:10 dilutions in sterile ddH_20 were made for both extraction samples using 5 µl of extract. Attempts were made to amplify regions containing diagnostic restriction sites for haplogroups A, C, D and X , the 9bp deletion (Haplogroup B) and 2 segments of HVI (bp16055 to bp16218 and bp16209 to bp16356)) using the PCR primers given in Table 1 and to digest the PCR products with restriction enzymes that recognize restriction sites diagnostic for each of these haplogroups (also given in Table 1).

Results:

RSM

The extraction blank (XN-1), phenol-chloroform extractions and 1:10 dilutions of the P/C extractions failed to amplify for the HVI segment from bp16209 to bp16356 (figure 2a). A single amplification of the HVI segment from bp16209 to bp16356 (see lane #2 in figure 1b) using 3µl of template purified using the Yang et al (1998) protocol was successful. Sequencing of this fragment performed at DBS sequencing center (UC Davis) indicated a single deviation from the Anderson (1981) reference sequence, a C to T transition at bp16223. A single amplification of the HVI segment from bp15938 to bp16139 using 3µl of template purified using the Yang et al (1998) protocol was also

successful. Sequence of this fragment performed at the DBS sequencing center (UC Davis) indicated a single deviation from the reference sequence, a G to A transition at bp16129. The G to A transition at bp16129 and the C to T at bp16223 are shared by one researcher (FAK) who had previously come in contact with this sample and is, therefore, presumed to represent contamination introduced during the preliminary analysis of the Kennewick sample. It is possible that rendering samples into powder increases the likelihood of contaminating the samples with the researchers own DNA.

JAE

Fragments containing the diagnostic markers for haplogroups A, B, C, D, and X and a segment of HVI (bp16055 to bp16218), as well as dilutions of these fragments, failed to amplify from the Kennewick extractions WAJA and WAJB (and extraction blanks for all reagents were clean). A single amplification of the HVI fragment from bp16209 to bp16356 using the undiluted extraction WAJA was successful. The sequence of this fragment generated at the DBS sequencing center (UC Davis) was identical to the reference sequence (Anderson et al 1981) as was that of JAE. Subsequent attempts to amplify this region were not successful and it was assumed that the former sequence represented contamination of the sample by JAE.

To test if DNA had been lost in the decalcification step, the EDTA washes were extracted using a phenol/chloroform (p/c) method as described above. A portion of the p/c extraction was subsequently purified using the modified Yang et al. (1998) protocol. No PCR products could be amplified from these extractions providing no evidence that any DNA had been lost during decalcification of the sample.

Conclusion:

No DNA suitable for PCR amplification could be extracted from the Kennewick samples studied. Thus, no conclusion regarding its ethnic ancestry or cultural affiliation based on DNA can be made. The source of all DNA that could be amplified in our studies was hypothesized to be one of two persons (FAK and JAE) who participated in the DNA analysis. Inhibitors were judged not to have interfered with amplifications because 1) primer-dimer formation occurred, 2) serial dilutions of PCR products did not lead to successful amplification and 3) mtDNA was successfully amplified from (i.e. was not inhibited by) a (50:50) mixture of a known positive sample and each of the PCR products. Since this particular sample from the Kennewick remains is likely to be the most optimal for extraction of DNA for analysis, it is unlikely that further analysis of other elements (e.g. teeth or a much larger portion of bone) would be successful.

This outcome might seem somewhat surprising since the collagen content of the samples analyzed was relatively high, given their great age, and human remains of comparable, even greater, age have yielded adequate DNA for analysis. However, there is very little data suggesting that the presence of protein in a given sample of bone is closely

correlated with its DNA content (the consensus of opinion of scientists attending the June, 2000 (biannual) Ancient DNA Conference in Manchester, England, was that such a correlation is, at best, low) and conditions of preservation of DNA in different samples of human remains varies substantially, largely independent of their age. It is significant that samples studied during 2000 were less contaminated (e.g., figure 2a) than those studied during 1996 (e.g., figure 1a). This is probably due to changes in methodology during the interim designed to reduce opportunities for contamination of ancient DNA with modern DNA. In fact, it is possible that methods developed in the near future could be successful in extracting suitable DNA for analysis from the Kennewick remains.

DOI 10002

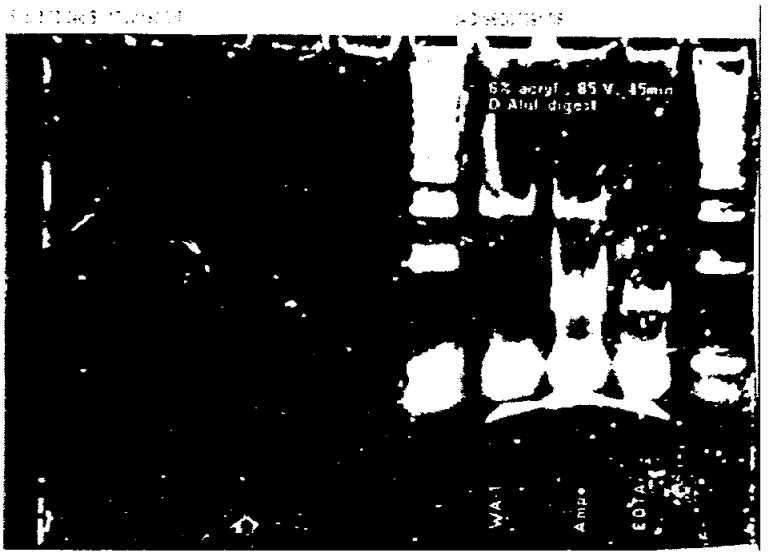
Table 1: Primers used for PCR amplification

target region	Primer Label	Primer Sequence (5'-3')	Annealing temp.	Fragment Length	Notes
A	L611	acctctcaaagcaatacactg	55	175bp	+HaeIII np663=A
	H743	gtgcttgatgcttgttccttttg			
B	L8215	acagtttcatgcccatcgtc	55	122bp (undeleleted)	+ 9 bp deletion (Deleted=B)
	H82197	atgctaagttagctttacag			
C	L13256	atcgtagccttctccacttc	55	186bp	-HincII np13259=C /+AluI np13262=C
	H13397	tcctcctatttttcgaatatctt			
D	L5129	ctactaccgcattcctactactcaac	55	110bp	-AluI np5176=D
	H5190	gggtggatggaattaagggtgt			
X	L14440	ctgacccccatgcctcagga	54	194bp	+AccI np 14465=X
	H14591	ctaagccttctcctatttatgg			
HVI	L15938	ccttttttccaaggacaaatcag	53	223bp	Control Region For sequencing
	H16139	tactacaggtggtcaagtat			
HVI	L16055	gaagcagatttgggtaccac	53	204bp	Control Region For sequencing
	H16218	tgtgtgatagttgagggttg			
HVI	L16209	ccatgcttacaagcaagt	51	186bp	Control Region For sequencing
	H16356	gtcatccatggggacgagaa			

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

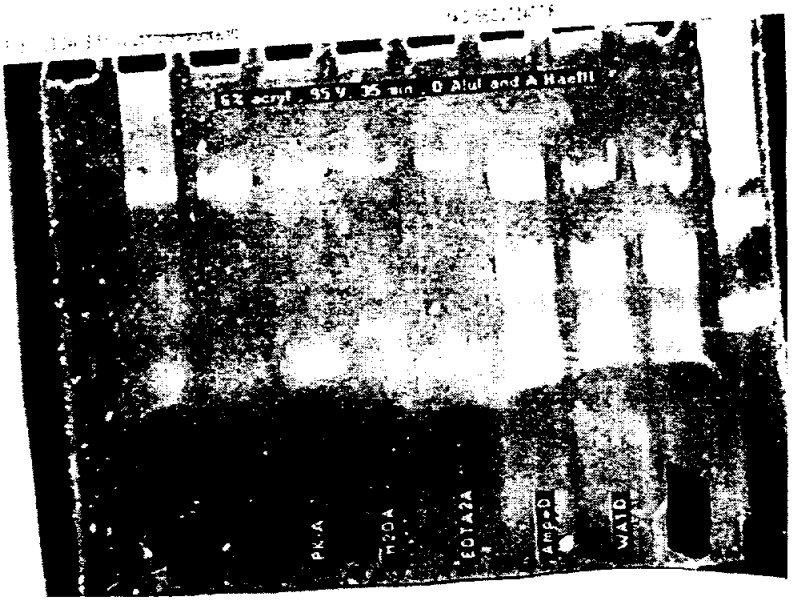
Figure 1. Preliminary analysis of restriction site characteristic of Native American haplogroup D in the second amplification of the first extraction (a), in the first amplification of the second extraction (b) and in further amplifications of both extractions (c), as described in the text. Note bands of primer-dimer below digestede portion of PCR fragments.

a.

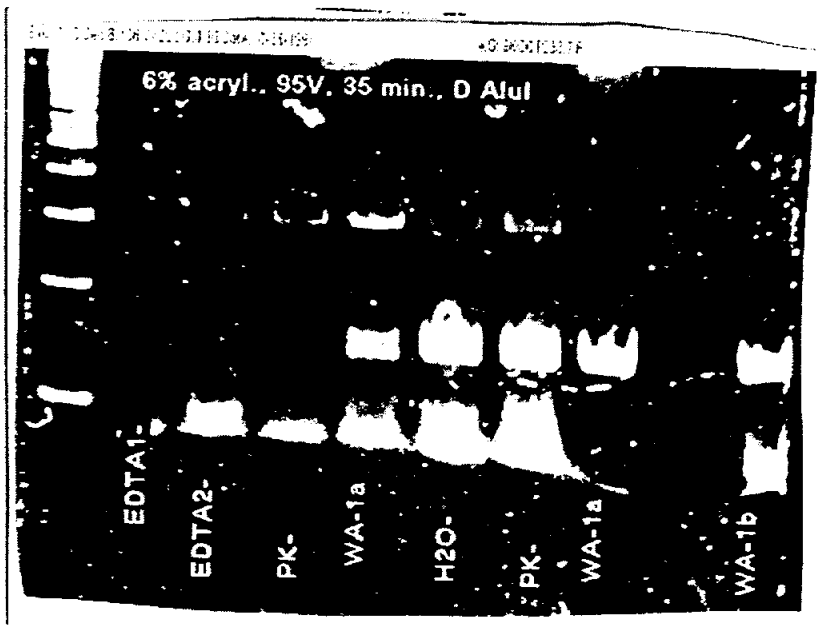


DOI 10004

b.


PK-A
H2OA
EDTA2A
Amp-D
WA1D

C.


6% acryl., 95V, 35 min., D AluI
EDTA1-
EDTA2-
PK-
WA-1a
H2O-
PK-
WA-1a
WA-1b

DOI 10005

Figure 2. Amplification of a portion of the HVSI section of the mitochondrial DNA control region of the Kennewick remains:a) after phenol/chloroform extraction, b) after silica gel (Yang protocol) extraction. Note that all lanes in gel **a.**,below,are blank.

a.

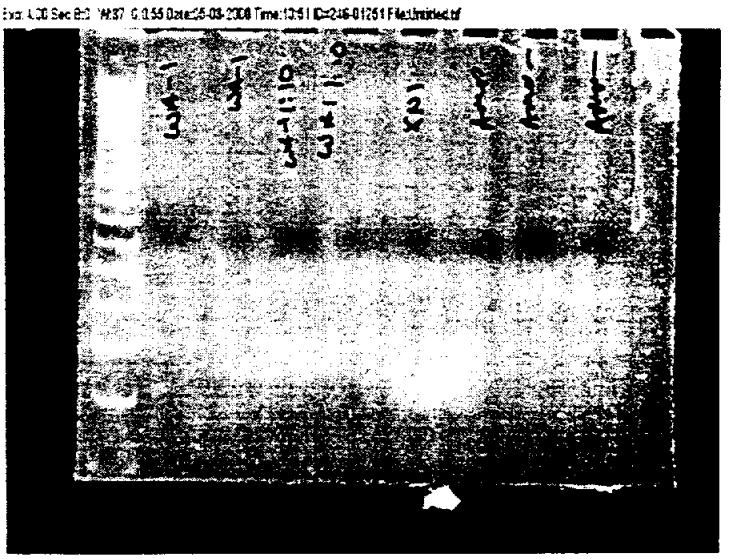
Amplification: Short d loop Date: 5/8/00

Number Cycles: 35 Holds: 68 95
3672

Centricon P/C Extract

	temp.	time
denature	95	30
anneal	51	30
extend	72	30

sample:	10mM dNTPs	50 uM primer 1	50 uM primer 2	ddH2O	10x buffer	20 mg/ml BSA	Taq	$MgCl_2$	DNaseI
per reaction									
1 UA-1									
2 WA-1									
3 WA-1 1:10									
4 WA-1 1:10									
5 YA1									
6 Amp-									
7 Amp-									
8 Amp -									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									
Totals									



b.

Amplification: short d loop

Number Cycles: 38

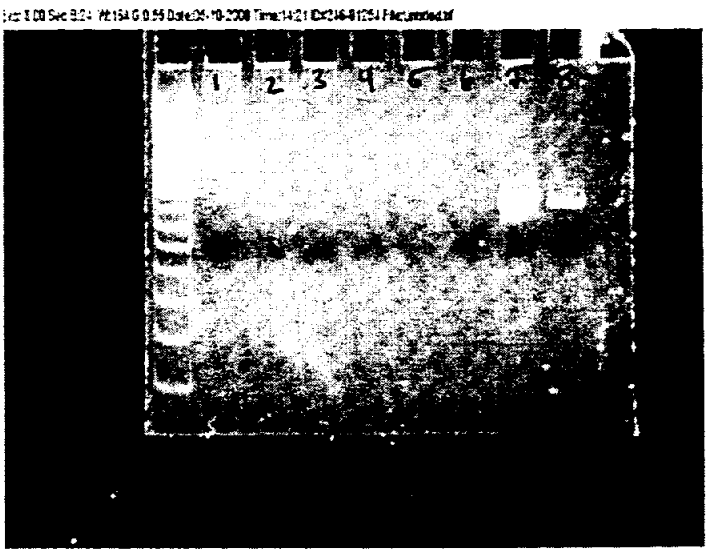
Date: 5/10/00

Holds: 6 6 95
3 6 72

	temp.	time
denature	95	30
anneal	51	30
extend	72	30

Yang clean up.

sample:	10mM dNTPs	50 uM primer 1	50 uM primer 2	ddH2O	10x buffer	20 mg/ml BSA	Taq	$MgCl_2$	DNaseI
per reaction									
1 WA-1 5d									
2 WA-1 3d									
3 "									
4 WA-1 1:10									
5 WA-1 1:10									
6 Amp -									
7 Amp +									
8 Amp +									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									
Totals									



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Anderson,S.,Bankier,A.T.,Barrell,B.G.,DeBruijn,M.H.L.,Coulson,A.R. et.al. 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457-465.

Yang, D.Y., End, B., Waye, J.S., Dudar, J.C. and S.R., Saunders. 1998. Technical Note: Improved DNA extraction from ancient bones using silica-based spin columns. American Journal of Physical Anthropology 105:539-543.

DOI 10008