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Ancient DNA Analysis of Human Populations

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ABSTRACT The use of ancient DNA (aDNA) in the reconstruction of population origins and evolution is becoming increasingly common. The resultant increase in number of samples and polymorphic sites assayed and the number of studies published may give the impression that all technological hurdles associated with aDNA technology have been overcome. However, analysis of aDNA is still plagued by two issues that emerged at the advent of aDNA technology, namely the inability to amplify a significant number of samples and the contamination of samples with modern DNA. Herein, we analyze five well-preserved skeletal specimens from the western United States dating from 800–1600 A.D. These specimens yielded DNA samples with levels of contamination ranging from 0–100%, as determined by the presence or absence of New World-specific mitochondrial markers. All samples were analyzed by a variety of protocols intended to assay genetic variability and detect contamination, including amplification of variously sized DNA targets, direct DNA sequence analysis of amplification products and sequence analysis of cloned amplification products, analysis of restriction fragment length polymorphisms, quantitation of target DNA, amino acid racemization, and amino acid quantitation. Only the determination of DNA sequence from a cloned amplification product clearly revealed the presence of both ancient DNA and contaminating DNA in the same extract.

Our results demonstrate that the analysis of aDNA is still an excruciatingly slow and meticulous process. All experiments, including stringent quality and contamination controls, must be performed in an environment as free as possible of potential sources of contaminating DNA, including modern DNA extracts. Careful selection of polymorphic markers capable of discriminating between ancient DNA and probable DNA contaminants is critical. Research strategies must be designed with a goal of identifying all DNA contaminants in order to differentiate convincingly between contamination and endogenous DNA. Am J Phys Anthropol 111:5–23, 2000.

New World indigenous groups were first assayed for mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs) and deletion/insertion events by Douglas C. Wallace and coworkers in the 1980s. These investigators defined four haplogroups, each based on a single RFLP or deletion, that were present at varying frequencies in populations throughout the New World and were proposed to represent the founding haplotypes of the New World (Torroni et al., 1992). The RFLP/deletion data were influential in shaping future molecular studies of indigenous groups in the Ameri-

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cas, and thousands of New World individuals have now been assayed for mitochondrial RFLP/deletions (e.g., Ward et al., 1991, 1993; Torroni et al., 1993; Kolman et al., 1995; Kolman and Bermingham, 1997; Merriwether et al., 1995). Varying frequencies of the four haplotype classes have been used to support or refute the controversial three-wave hypothesis of New World colonization (Greenberg et al., 1986; Torroni et al., 1992; Kolman et al., 1996).

With the advent of polymerase chain reaction (PCR) technology in the field of ancient DNA (aDNA), the application of these markers to prehistoric populations would seem obvious, though perhaps not as straightforward as originally thought. The RFLPs and deletion defined by Wallace and coworkers represent only a subset of all mitochondrial polymorphisms currently assayed in contemporary New World indigenous populations and, furthermore, contemporary New World populations carry only a fraction of the mitochondrial variation present worldwide. The diagnostic RFLP/deletions do not define monophyletic haplotype clusters in populations outside the New World, even in ancestral Asian populations (Kolman et al., 1996). Since modern populations may carry less genetic variation than their ancestors or may be more distantly related to ancient populations than is currently recognized, it may be a dangerous strategy to assay prehistoric populations for a restricted set of polymorphisms that have been culled from contemporary populations. To assay ancient specimens for only a few diagnostic markers with the justification of damaged aDNA and commensurate increase in time required for aDNA analyses is to invite incorrect haplogroup assignments.

Identification of contamination and authentication of results in aDNA studies rely on the ability to discriminate between ancient populations and DNA contamination. Numerous polymorphisms have been identified which distinguish New World populations from European populations (it is generally assumed that DNA contamination is likely to be of European origin), although the majority of these markers occur as mitochondrial D-loop haplotypes that are not detected by RFLP analysis but must be determined by DNA sequence analysis. The reduced mitochondrial diversity of New World populations (Kolman et al., 1996) and the large comparative database of mitochondrial sequences worldwide permit the straightforward identification of outlier haplotypes in New World populations. The highly unusual confluence of these characteristics makes the New World an ideal case for detection of contaminants. Ancient DNA studies wherein the prehistoric population and likely source(s) of contamination are genetically similar will be much more difficult, if not impossible, to conduct (e.g., Richards et al., 1995).

We present results on five well-preserved skeletal specimens from the western United States dating from 800–1600 A.D. In addition to D-loop sequence determination and mitochondrial RFLP/deletions, numerous analyses were performed in order to assay genetic variation and detect contamination in these samples. In sum, our data demonstrate that: 1) negative extraction and PCR controls do not ensure lack of contamination, 2) the presence of intact and cut bands in a restriction endonuclease digestion does not necessarily signify an incomplete digest but may reveal the presence of two DNAs, one of which is likely to be a modern contaminant, 3) DNA sequence determination of cloned amplification products is necessary to give a clear picture of the nature and extent of contamination in a sample, 4) methods such as amino acid racemization and DNA quantitation are minimally useful in detecting contamination, and 5) assaying the complete set of diagnostic RFLP/deletions can provide corroborative support for haplotype identification based on DNA sequence determination.

MATERIALS AND METHODS

Samples

Five skeletons, excavated at four sites in the Plains region, were investigated. The five specimens span 800 years in time, with samples 1 and 5 dating to the late Woodland period (sample 5 has an uncalibrated radiocarbon date of 760 ± 50 AD [TO-2189]). Samples 2–4 date to the period 1200–1600 AD. A femoral fragment from each specimen

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was used in all DNA and protein extractions. DNA extractions were saved from a previous natural abundance stable isotope study. These skeletons were collected prior to the use of molecular biological techniques and the attendant precautions now established to reduce contamination during excavation and curation. The results reported herein are considered representative of the many aDNA studies currently being pursued on similar collections.

Contamination precautions

No DNA-based studies of modern human populations were ever conducted in the building in which these experiments were executed. Positive PCRs were never performed. Several "no DNA" PCRs were performed in each series of reactions. Cross-contamination between samples was considered unlikely due to the low levels of amplifiable aDNA present in the samples. In retrospect, the contaminants also appeared to be present at very low levels and not prone to cross-contamination, given that only one out of eight contaminating sequences was found in more than one sample. All pre-PCR protocols were performed in a laboratory designed with positive hepa-filtered air pressure and dedicated to aDNA work. PCR and post-PCR analyses were conducted in separate wings of the building. All post-extraction manipulations were conducted by C.J.K. All reagents, including sterile water, were purchased as ready-made solutions (Amresco, Inc., Solon, OH). Disposable laboratory coats, gloves, filter tips, dedicated pipetmen, and disposable laboratory ware were used throughout the analyses. Benches and equipment were frequently treated with a 20% bleach solution. Sterile water (Amresco, Inc.) was aliquoted and irradiated by placing the tubes directly on a light source of 254 nm for 30 min (Sarkar and Sommer, 1990). All PCR reagents and primers were also aliquoted and irradiated at 254 nm for 30 min. During PCR setup, all tubes and the reaction cocktail minus DNA and enzyme were irradiated at 254 nm for 20 min prior to addition of DNA and enzyme.

DNA extraction and purification

Powdered bone, for DNA extraction, was generated by grinding bone fragments under liquid nitrogen in a Spex mill (Spex Industries, Inc., Edison, NJ). The bone powder was then placed in 6,000–8,000 molecular weight cut-off (MWCO) (Spectrum Medical Industries, Los Angeles, CA) dialysis tubing and immersed in 0.5 M EDTA, pH 7.3, at 4°C until complete decalcification had occurred. Due to the high collagen content of the skeletal material and the low temperature used for decalcification, complete decalcification required approximately 2–3 weeks. The EDTA extract was washed extensively with doubly distilled water (ddH_2O) and concentrated by passage through a YM30 filter (Amicon, Inc., Beverly, MA). Each filter was washed five times with ddH_2O, and the washes were processed as blanks in order to detect cross-contamination between YM30 filtrations. To decontaminate the filtration unit, it was boiled between samples.

Experiments were performed to test the efficacy of bleach or EDTA pretreatment in reducing DNA contamination of the skeletal material. Bleach treatment involved immersing the powdered bone in 20% bleach for 2 min followed by extensive ddH_2O washing. The EDTA protocol consisted of a 2-day treatment with 0.5 M EDTA at 55°C. Following each pretreatment, DNA was extracted by immersing the powdered bone in 0.5 M EDTA at 4°C until complete decalcification occurred.

A variety of purification protocols were tested, including proteinase K digestion (Sigma, St. Louis, MO), organic extraction of proteins with phenol and/or chloroform: isoamyl alcohol (Amresco, Inc.), ethanol precipitation of nucleic acids, filtration through Centricon-30 concentrators (Amicon, Inc.), and resin-based purification (Magic Megaprep DNA Purification System, Promega, Madison, WI).

Optimization of the extraction and purification protocol is presented below.

Inhibition assay

An assay to detect inhibition of the PCR enzyme was performed on all DNA extracts and extract dilutions up to 1:100. The 10-μl

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TABLE 1. Polymorphic regions and corresponding amplification conditions

Region	Primer[1]	Sequence	Annealing temperature	Length (bp)	Reference
Control region I	L16210	CCCATGCTTACAAGCAAGTA	51°C	132	This study
	H16301	TGGCTTTATGTACTATGTACTG			This study
Control region I	L16210	See above	50°C	184	This study
	H16356	GTCATCCATGGGGACGAGAA			Handt et al., 1996
Control region I	L16210	See above	51°C	239	This study
	H16410	GCGGGATATTGATTTCACGG			Handt et al., 1996
Control region I	L16210	See above	51°C	327	This study
	H16498	CCTGAAGTAGGAACCAGATG			Ward et al., 1991
COII/tRNA^{Lys}	L08215	ACAGTTTCATGCCCATCGTC	55°C	121	Wrischnik et al., 1987
Intergenic deletion	H08297	ATGCTAAGTTAGCTTTACAG			Wrischnik et al., 1987
*Hae*III:bp 663	L00602	TGTAGCTTACCTCCTCAAAGC	49°C	164	This study
	H00725	TTGATCGTGGTGATTTAGAGG			This study
*Alu*I:bp 5,176	L05170	CCTACTACTATCTCGCACCTG	49°C	132	This study
	H05261	GTGAATTCTTCGATAATGGCC			This study
*Dde*I:bp 10,394,	L10308	CCATGAGCCCTACAAACAACTAACC	55°C	201	Kolman et al., 1996
*Alu*I:bp 10,397	H10459	GTAAATGAGGGGCATTTGGTAAATAT			Kolman et al., 1996
*Alu*I:bp 13,262	L13232	CGCCCTTACACAAAATGACATCAA	49°C	207	Ward et al., 1991
	H13393	TCCTATTTTTCGAATATCTTGTTC			Stone and Stoneking, 1998
*Hae*III:bp 16,517	L16495	TAGCTAAAGTGAACTGTATCC	49°C	113	This study
	H16567	GGTGATAGACCTGTGATCCAT			This study

[1]Numbers in the primer designations identify the 3' ends of the primer according to the human reference sequence (Anderson et al., 1981).

assay consisted of 0.5 µl control template lambda DNA (PE Applied Biosystems, Foster City, CA), 0.5 µl each of primers PC01 and PC02 (PE Applied Biosystems), variable amounts of sample DNA extract, and reaction components as described below, except that bovine serum albumin was not added and 0.4 unit AmplitaqGold (PE Applied Biosystems) was used. Thermal cycler conditions consisted of an initial 12-min incubation at 95°C, followed by 23 cycles of 30 sec at 94°C, 30 sec at 53°C, and 45 sec at 72°C. PCR products were analyzed by electrophoresis on 2% agarose gels. Magnitude of inhibition was determined by a visual comparison of sample DNA PCR to a control PCR lacking sample DNA.

DNA amplification

Amplifications were performed in GeneAmp buffer (PE Applied Biosystems, 1× = 10 mM Tris, pH 8.3, 50 mM KCl), 1.75 mM $MgCl_2$ (PE Applied Biosystems), 200 µM of each dNTP (PE Applied Biosystems), 20 µg/ml bovine serum albumin (BSA; Sigma), and 1 µM of each primer (Integrated DNA Technologies, Inc., Coralville, IA). Spermidine (400–800 µM) was added to facilitate amplification of DNA samples that did not amplify initially. For each 10-µl reaction, 1.25 units AmplitaqGold (PE Applied Biosystems) were added. Reaction volumes varied between 10–40 µl. Table 1 lists the primers used and the corresponding DNA sequences, annealing temperatures, and amplification product lengths. All reagents except DNA and enzyme were combined into a reaction cocktail and, along with reaction tubes, were irradiated at 254 nm for 20 min. Following an initial 12-min incubation at 95°C to activate the enzyme, 50 cycles of PCR, 30 sec at 94°C, 30 sec at the relevant annealing temperature, and 45 sec at 72°C, with a final extension cycle at 72°C for 5 min, were performed in a Perkin Elmer 480 Thermocycler (Foster City, CA) using a CyclerMate (Biologic Engineering, Inc., Shelton, CT) in lieu of paraffin oil. A Perkin Elmer 9600 Thermocycler and Idaho Technology 1605 Air-Thermo Cycler (Idaho Falls, ID) were also tested.

Restriction digests of PCR products with 10–20 units of the appropriate restriction enzyme (New England Biolabs, Beverly, MA) were performed using the recommended buffer and incubated overnight at 37°C. Digestion products were analyzed by electrophoresis on 2% agarose gels. Amplification products covering the 9-bp region were analyzed on 4% Metaphor (FMC BioProducts, Rockland, ME) gels.

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Optimization of the DNA amplification reaction is presented below.

DNA quantitation

DNA quantitation was performed as described in Handt et al. (1996). A competitor construct containing a deletion in the mitochondrial D-loop region, constructed by Handt et al. (1996), was used. DNA concentration of the deletion plasmid was determined by optical density measurement at 260 and 280 nm. D-loop control region I primers listed in Table 1 were used to produce four PCR products of increasing size: 132 bp, 184 bp, 239 bp, and 327 bp. Reaction components were as described above. Thermal cycler conditions consisted of an initial 12-min incubation at 95°C followed by 45 cycles of 50 sec at 94°C, 50 sec at 48°C, and 50 sec at 72°C, with a final extension cycle at 72°C for 5 min.

Cloning and sequencing

PCR products were separated by gel electrophoresis, excised as agarose bands, purified using a GeneClean II kit (Bio 101, Inc., La Jolla, CA), and resuspended in approximately 10 µl ddH_2O. PCR products were then either sequenced directly or ligated into pCR-Script SK(+) and cloned in XL1-Blue MRF' cells (Stratagene, La Jolla, CA). Direct sequencing was performed using PCR primers and a DNA Sequencing Kit, FS version (PE Applied Biosystems). In order to generate PCR clones, approximately 5 µl of purified PCR product were cloned using the PCR-Script Cloning Kit (Stratagene). Plasmid DNA from clones was prepared using a modified alkaline lysis/polyethylene glycol precipitation plasmid miniprep protocol (PE Applied Biosystems). Sequencing was performed using M13 forward and reverse primers (Integrated DNA Technologies, Inc.) and a DNA Sequencing Kit, FS version (PE Applied Biosystems). All sequencing reactions were analyzed on a 373 DNA Sequencer (PE Applied Biosystems). DNA sequence data were collected between bp 16,211–16,400 of the mitochondrial D-loop control region I, with the exception of six clones which contained a 132-bp PCR product and were sequenced between bp 16,211–16,300 (numbering according to Anderson et al., 1981). Both light and heavy strands were sequenced for all clones.

Amino acid racemization

Approximately 10 mg of powdered bone or insoluble collagen were hydrolyzed by placing samples in sealed tubes containing 6 N HCl at 110°C for 20 hr. Insoluble collagen was prepared by complete decalcification of powdered bone samples in 0.5 M EDTA, pH 7.4, at room temperature. Trifluoroacetic isopropyl ester derivatives of the hydrolyzed amino acids were prepared using a TFA-IPA Amino Acid Derivatization kit (Alltech Associates, Inc., Deerfield, IL). Chiral separation of the amino acid enantiomers was performed on a Carlo Erba Mega Series 5300 gas chromatograph (Thermo Separations, Riviera Beach, FL) with a Chirasil-Val capillary column (Alltech) and flame ionization detection. To determine the extent of racemization due to the experimental procedure, a fragment of fresh, whole cow bone was analyzed. Powdered bone samples were available only for samples 1–3.

Individual amino acid determination

Approximately 10 mg of powdered bone were hydrolyzed by placing samples in sealed tubes containing 6 N HCl at 110°C for 20 hr. Hydrolysates were dried and resuspended in a dilute HCl solution, pH 2.0. Samples were analyzed on a Model 2000 amino acid analyzer liquid chromatograph (St. John's Associates, Beltsville, MD) using a cation exchange column (St. John's Associates). Eluted amino acids were derivatized post-column using *o*-phthaldehyde and flouresently detected. Premixed amino acid standards were used for quantitation (Standard H, Pierce, Rockford, IL). Sufficient bone material for amino acid determination was available only for samples 1–3.

RESULTS

The studied skeletal material was excavated in the middle of the twentieth century and represents part of a large population that spanned almost 2,000 years of human habitation. The geologic condition of the excavation sites was cool and dry, and the skeletal material was in uniformly excellent condition. Scanning electron micrographs of

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bone cross sections confirmed the excellent preservation of morphological characteristics such as collagen fibers and nonmineralized, open Haversian canals (data not shown). Colson et al. (1997) proposed that histological preservation of skeletal material is the best indicator of DNA amplifiability. The skeletal material was generally a pale color similar to fresh bone, with little of the dark discoloration found in buried bones subjected to frequent perfusion by groundwater and accompanying compounds. Soil-derived humic acids result in brown staining on bone and are thought to be at least partially responsible for the inhibitors present in archaeological skeletal material.

Nucleic acids were extracted from 19 specimens. In general, it was possible to obtain appropriately-sized PCR products from all DNA samples. However, it became clear early in the study that contamination of the aDNA samples with modern DNA was a persistent problem. Extraction controls and PCR controls were negative for all reported results, so the cause was not simple contamination of reagents or laboratory ware. Only select specimens produced DNA samples with evidence of modern contamination and, in these samples, the contamination was not consistent in every amplification reaction.

When present, contamination was observed as two bands on an agarose gel when a single New World-specific polymorphism was assayed. For example, sample 2 exhibited the *Alu*I site at bp 13,262 that is diagnostic for haplogroup C. However, agarose gel electrophoresis revealed two bands in sample 2 corresponding to the presence and absence of the *Alu*I recognition site (Fig. 1). The two bands are unlikely to reflect a partial digestion by the *Alu*I restriction enzyme since PCR products from all other samples (a total of five samples were assayed for *Alu*I:13,262) digested completely. Instead, the two bands most likely correspond to two sources of DNA, one an ancient group C haplotype and the other a modern variant lacking the *Alu*I site. In addition, samples 3 and 4 exhibited the 9-bp deletion that is diagnostic for haplogroup B. However, they both showed a band corresponding to the undeleted form of this marker in addition to the deletion (Fig. 2). The same

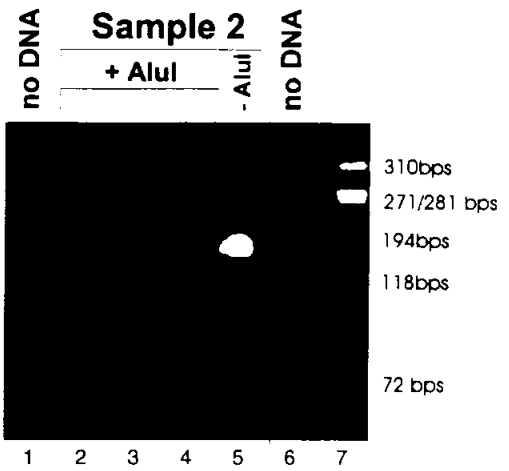

Fig. 1. Ethidium bromide-stained agarose gel showing the results of PCR analysis of the *Alu*I/13262 site in sample 2. **Lanes 1** and **6** depict "no DNA" PCR controls, **lanes 2–4** depict three amplification reactions using varying amounts of the same sample 2 DNA extract followed by *Alu*I digestion, and **lane 5** is an undigested PCR of sample 2. DNA size markers were run in **lane 7.**

result was obtained with multiple DNA extractions from the two samples. In this case, there was no possibility of a partial digest accounting for the two bands, and the cause was clearly two sources of DNA.

At this point, five skeletal specimens were chosen for further analysis based on the following criteria: dating consistent with pre-European contact, reproducible amplifiability of DNA extracts, and representation of the full range of contamination from 0–100%.

Optimization of DNA extraction and purification protocol

A variety of extraction and purification protocols were tested which involved varying degrees of decalcification of the skeletal material and subsequent purification using combinations of proteinase K digestion, organic extraction of proteins, ethanol precipitation of nucleic acids, Centricon filtration, and resin-based purification. The results of these experiments can be summarized as follows: 1) More complete decalcification resulted in stronger aDNA PCR signals and weaker contaminating DNA signals (when contamination was present). 2) Minimal modification of the crude DNA extracts, i.e.,

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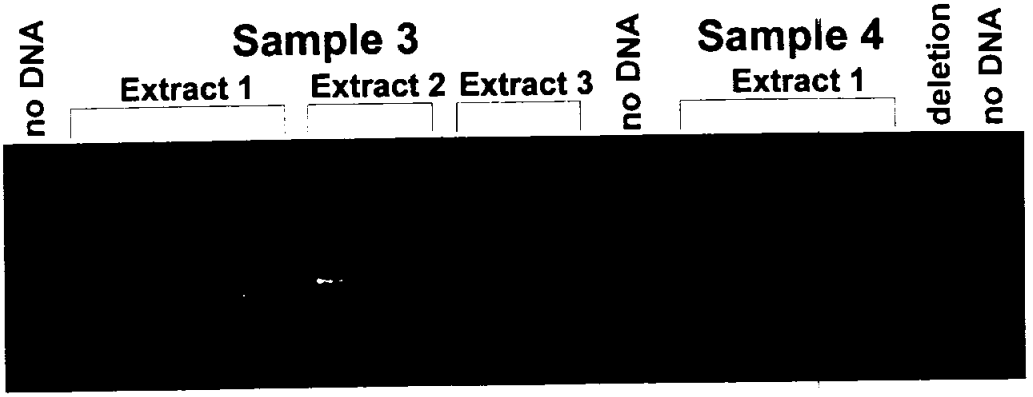

Fig. 2. Ethidium bromide-stained agarose gel, showing the results of PCR analysis of the 9-bp deletion in samples 3 and 4. Independent PCRs using varying amounts of DNA from three sample 3 extracts and one sample 4 extract are presented. Controls include three "no DNA" PCRs and a PCR of an individual with the 9-bp deletion (positive control PCR was performed at the Smithsonian Tropical Research Institute in Panama).

minimal purification, also resulted in stronger aDNA PCR signals and weaker contaminating DNA signals.

During the initial days of bone decalcification, the highest quantity of inhibitory material was released, and this was visualized as a blue-white smear on an agarose or polyacrylamide gel prior to staining with ethidium bromide. Conversely, a greater quantity of aDNA was released subsequent to this period, i.e., after the first week of bone decalcification. In a separate experiment using skeletal material from Easter Island, a DNA extract collected during the first week of decalcification was visibly colored and produced no successful amplifications, whereas an extract collected during weeks 2 and 3 of decalcification was nearly colorless and generated successful amplifications (Kolman et al., in press). Complete decalcification of skeletal material may be necessary only for older specimens, as Fisher et al. (1993) reported that no decalcification was necessary for bones up to 125 years old.

Additional purification of the DNA extract subsequent to the initial YM30 filtration and concentration was found to be unnecessary and, in some cases, disadvantageous. For example, a protocol using Proteinase K digestion, organic extraction, and ethanol precipitation resulted in DNA which generated a weaker aDNA PCR signal and a stronger contamination signal relative to amplification of an unpurified extract (data not shown). The most dramatic effect was seen with resin-based purification (Magic Megaprep DNA Purification System, Promega), which resulted in exclusive recovery of contaminating DNA. This result may be due to the altered binding of aDNA that is cross-linked to proteinaceous material and passes through a column prepared with the resin, allowing preferential purification of the contaminating DNA. The inability of DNA-protein complexes, such as those found in ancient DNA extracts, to interact properly with DNA-binding resins provides a cautionary note for purification protocols based on these resins, e.g., silica-based purifications (Höss and Pääbo, 1993).

Two methods for eliminating surface DNA contamination from skeletal material were also investigated. 1) Previous researchers have proposed that skeletal material be treated with a bleach solution prior to DNA extraction (e.g., Richards et al., 1995). The rationale is that this treatment will preferentially damage and destroy superficial DNA such as that acquired through investigator handling. Powdered bone from sample 3 (which exhibited deleted and nondeleted forms of the 9-bp region) was subjected to a 2-min immersion in 20% bleach followed by extensive washing with ddH_2O. 2) Based on our results showing preferential extraction of inhibitory material during the initial

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stages of decalcification, a 2-day pretreatment with 0.5 M EDTA at 55°C was also tested for efficiency in removing contamination. Bone powder treated by both procedures was then placed in 0.5 M EDTA at 4°C to extract DNA. Although both treatments decreased the incidence of the nondeleted 9-bp band in sample 3, neither treatment completely eliminated this evidence of contamination. With no pretreatment, 67% (4/6) of PCRs generated both deleted and nondeleted 9-bp bands. Following either Clorox or EDTA treatment, only 25% (1/4) of PCRs generated both bands. In all other PCRs, only a single, deleted 9-bp band, diagnostic for haplogroup B, was generated. The inability to eliminate contamination derived from specimen handling is not unexpected, due to the porous nature of bone material that provides easy access for contaminating DNA molecules deposited during handling.

Optimization of DNA amplification

Approximately 1–5 µl of DNA extract were typically used in DNA amplifications. The DNA extracts were not inhibitory as determined by lambda DNA amplification and, thus, DNA dilutions were not necessary for successful amplification.

Irradiation of all reaction components, including tubes, ddH_2O, buffer, $MgCl_2$, BSA, dNTPs, and DNA primers, immediately prior to addition of DNA and enzyme (Sarkar and Sommer, 1990), was necessary for consistently negative extraction and PCR controls. Three sets of irradiation conditions were tested: 312 nm for 20 min, 312 nm for 40 min, and 254 nm for 20 min. Only irradiation at the more intense wavelength of 254 nm for 20 min successfully eliminated all signals in negative controls and significantly reduced the intensity of the contaminating DNA PCR when contamination was present. Additionally, commercially obtained DNA primers were frequently found to be contaminated and were routinely "decontaminated" by irradiation at 254 nm for 20 min. This practice did not destroy the utility of the DNA primers despite the frequent occurrence of neighboring thymines in the primer sequences and the potential for formation of ultraviolet (UV)-induced thymine dimers.

A "heat-soak" step (Ruano et al., 1992) at 95°C for 10–20 min prior to temperature cycling was essential for strong DNA amplification. This was achieved either by adding the primers, dNTPs, and enzyme after the "heat-soak" step, or by using AmplitaqGold DNA polymerase which required a 12-min 95°C incubation for full activity.

Low levels of spermidine facilitated amplification of certain DNA samples (Wan and Wilkins, 1993). The spermidine concentration was critical, because too much spermidine was found to inhibit the PCR. Optimal final concentrations of 400–800 µM spermidine were empirically determined. However, these spermidine concentrations inhibited amplification of DNA samples that amplified in the absence of spermidine. Therefore, DNA samples were first tested for amplification in the absence of spermidine, and those DNA samples with no amplification were tested again with spermidine.

Reamplification of a weak PCR product extracted from an agarose gel was unsatisfactory and consistently generated contamination. The contamination most likely derived from previously amplified DNA that was present in the electrophoresis equipment. Supporting this interpretation, successful amplification of human mitochondrial D-loop DNA was recovered from a piece of agarose in which no DNA had been electrophoresed.

Various thermal cycling machines were also tested. An air cycler (Idaho Technologies) never yielded successful amplification of the aDNA samples. In a comparison between Thermocyclers 480 and 9600 (Perkin Elmer, Idaho Falls, ID), the 480 model consistently yielded stronger aDNA PCR signals and weaker contamination PCR signals. Both results were supported by a separate study of DNA extracted from archival fish specimens (Kolman and Tuross, unpublished data).

RFLP analysis

Four haplogroups, labeled A, B, C, and D, were defined by Torroni et al. (1992, 1993) based on mitochondrial restriction/deletion polymorphisms, and were proposed to represent founding New World haplogroups. The diagnostic sites are listed in Table 2. These six polymorphisms were assayed in the five ancient samples and are presented in Table 3.

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TABLE 2. Mitochondrial DNA markers diagnostic for founding New World haplogroups

Site[1]	Haplogroup A	Haplogroup B	Haplogroup C	Haplogroup D
Restriction/deletion sites				
*Hae*III:663	1	0	0	0
*Alu*I:5176	1	1	0	0
*Dde*I:10,394	0	0	1	1
*Alu*I:10,397	0	0	1	1
*Alu*I:13,262	0	0	1	0
9-bp	2	1	2	2
Control region I polymorphisms				
16,217	T	C/C	T	T
16,223	T	C/C	T	T
16,290	T	T	C	C
16,298	T	T	C	T
16,319	A	G	G	G
16,325	T	T	C/t	C
16,327	C	C	T	C
16,362	C	T	T	C

[1] Diagnostic restriction/deletion sites were defined by Torroni et al. (1993), and diagnostic control region I polymorphisms were defined by Horai et al. (1993). Presence and absence of a restriction site are indicated by 1 and 0, respectively. One and two copies of the 9-bp repeat are indicated by 1 and 2, respectively. For control region I DNA sequence data, the defining sites are underlined. X/y indicates that X is the predominant nucleotide at that position, although Y occurs at a low frequency.

TABLE 3. Polymorphic restriction, deletion, and nucleotide sites of ancient DNA samples

	RFLP/deletion analysis of PCR products[1]							DNA sequence analysis of cloned PCR products[2]												
								D-loop polymorphic sites											New	
Sample	*Hae*III 663	*Alu*I 5,176	*Dde*I 10,394	*Alu*I 10,397	*Alu*I 13,262	9-bp 8,272/ 8,289	New World haplogroup	16217	16218	16223	16298	16319	16325	16362	16390	16391	16399	16402	World haplo- group	No. of clones
Reference	0	1	0	0	0	2		T	C	C	C	C	C	T	T	T	T	C	C	T			
Sample 1	0	1	1	1	1	2	C	.	.	T	.	.	.	C	.	.	C	T	.	.	C	4
Sample 2	0	1	1/0	1/0	1/0	2	C/nN	.	.	T	.	.	.	C	.	.	C	T	.	.	C	5
								.	.	**T**	.	.	.	**C**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	C	5
								.	G	.	.	.	.	.	nd	nd	nd	nd	nd	nd	nN	1
								C	.	T	.	.	.	.	nd	nd	nd	nd	nd	nd	nN	1
Sample 3	0	1	0	0	0	1/2	B/nN	C	.	.	.	.	.	.	.	.	.	.	.	.	B	3
								.	T	.	.	.	.	.	.	.	.	.	A	C	nN	3
								C	.	T	.	.	.	.	.	.	.	.	.	.	nN	3
								.	.	.	.	.	.	.	.	.	.	.	.	.	nN	2
								.	.	.	.	.	.	.	.	C	.	.	.	.	nN	2
Sample 4	0	1	0	0	0	1/2	B/nN	.	.	.	T	T	.	C	.	.	.	.	.	.	nN	4
								C	.	.	.	.	.	.	.	.	.	.	.	.	B	2
Sample 5	0	1	0	0	0	2	nN	.	.	.	.	.	.	.	.	.	.	.	.	.	nN	3
								.	.	.	T	.	.	.	.	.	.	.	.	.	nN	2

[1] Presence and absence of a restriction site are indicated by 1 and 0, respectively. One and two copies of the 9-bp repeat are indicated by 1 and 2, respectively. 1/0 or 1/2 signifies that two sets of bands were identified in the same sample.
[2] DNA sequences determined from direct sequencing of PCR products are shown in bold type. nd, not determined. DNA sequence of 132-bp PCR products was only determined between bp 16,210–16,300. nN, a non-New World haplotype.

Samples 1 and 2 exhibited the *Alu*I site at bp 13,262 that is diagnostic for haplogroup C. However, agarose gel electrophoresis revealed two bands in sample 2 corresponding to the presence and absence of the *Alu*I recognition site (Fig. 1). Sample 2 was also polymorphic for the *Dde*I site at bp 10,394 and the *Alu*I site at bp 10,397 (Table 3). The presence of these three sites is diagnostic for New World haplogroup C, while absence of these sites is most common in European populations. Sample 1 was invariant for the presence of *Dde*I:10,394, *Alu*I:10,397, and *Alu*I:13,262.

Samples 3 and 4 displayed the 9-bp deletion that is diagnostic for haplogroup B. However, they both showed a band corresponding to the undeleted form of this marker in addition to the deletion (Fig. 2). The same result was obtained with multiple DNA extractions.

Sample 5 displayed none of the diagnostic RFLP/deletion polymorphisms for New World founding haplogroups.

DNA sequence analysis

Diagnostic polymorphisms in the mitochondrial D-loop control region I have also been defined for New World founding haplogroups and are presented in Table 2, as

summarized in Kolman et al. (1996) from Horai et al. (1993). D-loop DNA sequences were determined for the five ancient samples directly from the amplified fragments and from cloned PCR products (unless otherwise stated, each set of clones was derived from a single amplification reaction). A summary of the cloned DNA sequences is presented in Table 3, with direct PCR sequences identified in bold type. DNA sequences are summarized such that sequences that appeared in only one clone and carried only a single mutation relative to another sequence in the sample were assumed to reflect DNA damage or polymerase errors and were not shown. Complete DNA sequences of all clones can be found in the Appendix.

All clones of sample 1 (n = 4) exhibited a single DNA sequence that contained all of the sites diagnostic for haplogroup C. Direct DNA sequence analysis of an amplification product confirmed the identical C haplotype sequence. No evidence of contaminating sequences was observed. These results were consistent with the RFLP/deletion analysis.

The DNA sequence for sample 2 was determined using cloned products from two independent PCRs. In one case, amplification was successful for the 239-bp product and, in the second case, amplification was recovered for only the smallest 132-bp product. All clones of the 239-bp fragment (n = 5) yielded sequences which corresponded to haplogroup C and were consistent with the identification of an *Alu*I site at bp 13,262 by RFLP/deletion analysis. Cloned sequence analysis of the 132-bp fragment resulted in multiple sequences, although the predominant sequence (n = 5) corresponded to haplogroup C. The second sequence (n = 1) showed a single, rare 16218:G mutation relative to the human reference sequence. The reference sequence is the most frequent haplotype in European populations (Richards et al., 1996). A third sequence from the 132-bp PCR (n = 1) contained two diagnostic polymorphisms for New World haplotypes but they define different haplotypes, i.e., 16217:C defines haplogroup B and 16223:C occurs in the three remaining New World haplotypes. This sequence may represent back-mutations, "jumping" PCR (Pääbo et al., 1990), polymerase error, template damage, or a combination of these events. Direct sequence analysis of 132-bp PCR products identified the C haplotype sequence.

The DNA sequence for sample 3 was determined from clones of five independent amplification reactions representing three 239-bp fragments and two 327-bp fragments. Five DNA sequences were recovered. One sequence (n = 3) contained the sites diagnostic for haplogroup B, and was consistent with the identification of the 9-bp deletion in sample 3 by RFLP/deletion analysis. Direct PCR sequencing also identified the B haplotype. None of the remaining sequences contained all of the diagnostic sites for any New World haplogroup. The second sequence (n = 3) was also identified by direct sequencing of a second PCR and contained both a rare polymorphism (16218:T) and a novel transversion (16328:A) that could reflect polymerase error or template damage. The third sequence (n = 3) carried the 16217:C polymorphism that is present in haplogroup B but did not contain the 16223:C site that also defines haplogroup B. The fourth sequence (n = 2) corresponded to the human reference sequence, which is the predominant haplotype in European populations (Richards et al., 1996). The 16311:C mutation present in the fifth sequence (n = 2) is found in populations worldwide, although always in combination with other mutations (Kolman et al., 1996; Richards et al., 1996; Torroni et al., 1996).

Sample 4 clones exhibited two distinct DNA sequences. The predominant sequence (n = 4) corresponded to one previously identified in European populations as haplogroup T (Torroni et al., 1996; C.J.K. is also haplogroup T). The second sequence (n = 2) contained the sites diagnostic for haplogroup B and was consistent with the identification of a 9-bp deletion in sample 4 by RFLP/deletion analysis. Direct DNA sequence analysis of PCR products identified the predominant haplogroup T sequence.

DNA sequence analysis of sample 5 identified two DNA sequences that differed by a single nucleotide. One sequence (n = 3) was identical to the human reference sequence

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(Anderson et al., 1981). The second sequence (n = 2) carried a C-to-T transition at nucleotide 16291 that has never been identified without accompanying mutations (Horai and Hayasaka, 1990; Horai et al., 1993) and most likely represents polymerase error or template damage. Direct sequence analysis of three independent PCRs identified the human reference sequence (Anderson et al., 1981). These results confirmed the RFLP/deletion analysis that also failed to identify any polymorphisms diagnostic for New World indigenous populations in sample 5. A radiocarbon date of 760 ± 50 AD was determined for sample 5, thus eliminating the possibility that it represented an intrusive burial. Sample 5 most likely experienced significantly more investigator handling than the other samples because of lesions suggestive of treponemal infection that were present on several of the bones.

DNA sequence analysis of cloned amplification products confirmed and clarified the results from the RFLP/deletion analysis. Samples 2–4, which exhibited two sets of agarose bands for different diagnostic restriction/deletion sites, displayed at least two D-loop DNA sequences, one consistent with a New World haplogroup and at least one non-New World haplotype that was likely of European origin.

DNA quantitation

The number of amplifiable copies of four variously sized DNA fragments in the DNA extracts was determined using a competition assay. A D-loop construct engineered with a deletion was utilized which allowed electrophoretic separation of the two sets of PCR products (Handt et al., 1996). All DNA copy numbers are presented as the quantity present in a 10-µl amplification reaction (Table 4). Sporadic, unsuccessful PCRs were recovered for samples 1 and 2 with primers for the 327-bp product, indicating that the DNA concentration was so low that stochastic effects played a large role in the outcome of the reaction (Krings, personal communication). These reactions were scored as containing zero copies of the large DNA fragment. Handt et al. (1996) proposed that a minimum of 10^3–10^4 copies was required for unambiguous and reproducible PCR results.

TABLE 4. Quantitation of mtDNA fragments

	No. of copies of variously sized PCR products[1]				Longest D-loop fragment amplified[2]
	132 bp	184 bp	239 bp	327 bp	
Sample 1	10^4–10^5	10^4–10^5	10^3–10^4	0	239 bp
Sample 2	10^4–10^5	10^4–10^5	10^3	0	239 bp
Sample 3	10^4–10^5	10^4–10^5	10^4–10^5	10^3–10^4	327 bp
Sample 4	10^5	10^4–10^5	10^4–10^5	10^4–10^5	327 bp
Sample 5	10^5–10^6	10^5–10^6	10^4–10^5	10^4–10^5	327 bp

[1] Numbers of amplifiable copies are given for the minimum volume of DNA extract required for successful DNA amplification in a 10-µl reaction.
[2] Length of longest D-loop PCR product which was cloned and analyzed.

DNA extracts of all five samples presented here contained this number of copies or more for all except the longest PCR product. A gradual reduction in number of copies was seen with increasing PCR product size and was consistent with the maximum size of D-loop fragment amplified in each sample.

Amino acid racemization

Racemization of aspartic acid (Asp), which has one of the fastest racemization rates of all amino acids, was assayed and is presented in Table 5 as a ratio of D-enantiomer to L-enantiomer (ratios are uncorrected for racemization caused by the experimental procedure, which was determined to be 0.030). D/L ratios were determined using both powdered bone (samples 1–3 only) and insoluble collagen. Using bone samples, Poinar et al. (1996) proposed that an Asp D/L ratio of 0.08 (uncorrected) represented the upper limit for extraction of amplifiable DNA. All D/L ratios reported for samples 1–5 are equal to or lower than this value, suggesting that all five samples are equally good candidates for aDNA studies.

Individual amino acid quantitation

Quantitation of 14 amino acids was determined in the three samples for which sufficient bone material existed. For all amino acids, the ancient samples showed levels comparable to those found in a fresh, whole cow bone sample (data not shown). The largest difference was seen in glycine, the amino acid present in the highest proportion due to its elevated levels in bone collagen; samples 1, 2, and 3 contained 78%, 102%, and 91% of the glycine, respectively, present

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TABLE 5. Amino acid analyses of ancient bone samples[1]

	Asp D/L ratio		Total protein (pmol amino acid/μg dry bone)
	Powdered bone	Insoluble collagen	
Sample 1	0.082	0.066	1,613
Sample 2	0.071	0.046	2,142
Sample 3	0.068	0.078	1,884
Sample 4	ND	0.065	ND
Sample 5	ND	0.077	ND
Fresh cow bone	0.030	ND	1,914

[1] ND, not determined.

in the modern sample. For comparative purposes, a collection of Panamanian Amerind bones that did not yield amplifiable DNA contained only 5–62% of the glycine present in a modern control (Kolman and Tuross, unpublished data). When summarized as total amount of protein, the ancient bone samples contained 84–112% of the quantity of amino acids found in the modern controls (Table 5). Good preservation of protein, as seen in the studied samples, suggests that similar levels of DNA preservation may be expected.

DISCUSSION

Identification of contamination has emerged as the single most critical issue in ancient DNA studies. Early, spectacular claims of successful DNA extraction from extremely old specimens, such as 17–20 million year (Myr) old *Magnolia* leaf fossils (Golenberg et al., 1990), 25–135 Myr old specimens preserved in amber (DeSalle et al., 1992; Cano et al., 1993), and 80 Myr old dinosaur bones (Woodward et al., 1994), have generally been disproved or cast into serious doubt (Sidow et al., 1991; DeSalle et al., 1993; Lindahl, 1993; Austin et al., 1997a). Later authors, using relatively simple methods, were able to detect contamination in the early studies, such as the phylogenetic analysis by Hedges and Schweitzer (1995) of proposed dinosaur DNA which identified it as modern human contamination. Current occurrences of contamination are subtler and more difficult to detect. Standard precautionary measures such as negative extraction and PCR controls, multiple extractions, and "clean" rooms, while necessary, are insufficient and did not identify the contamination reported here. The contaminated specimens studied here appear to contain two populations of amplifiable DNA molecules, one ancient in origin and the other of modern origin (assuming no heteroplasmy). Both DNAs were present at extremely low concentrations and were unable to efficiently amplify alone. Both DNAs were needed to increase the DNA concentration to a critical level, at which point they began competing for reagents (Handt et al., 1994).

Contamination of archaeological material and the complex interplay between endogenous ancient DNA and modern contamination reported here are unlikely to represent a unique situation. Numerous cases exist in the published literature that indicate similar problems have been previously encountered, only some of which were identified as contamination. The recent determination of the Neandertal mitochondrial D-loop control region DNA sequence by Krings et al. (1997) was obtained through sequence analysis of cloned PCR products. Two distinct sets of DNA sequences were reported, one significantly different from modern human sequences and presumed to be Neandertal in origin, and the other identical to the human reference sequence (Anderson et al., 1981) and presumed to be modern contamination. A second example of contamination is provided by Handt et al. (1996), who identified deleted and nondeleted forms of the 9-bp region in two out of three 600-year-old New World skeletal samples. Recently, studies have attempted to use human remains to identify infectious disease agents, such as *Mycobacterium tuberculosis, Yersinia pestis,* and *Bacillus anthracis* (pathologic agents for tuberculosis, bubonic plague, and anthrax, respectively; Salo et al., 1994; Drancourt et al., 1998; Jackson et al., 1998). However, these studies all suffer from the common practice of inclusion of a positive PCR control that, in the reported studies, yielded identical DNA sequences to the putative ancient DNA extracts. The finding of identical sequences in an archaeological specimen and the control DNA sample precludes convincing proof that ancient DNA was extracted and analyzed. Conscientiousness and complete disclosure of results make it possible to assess the types and extent of

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contamination that may be present in the majority of aDNA studies. Reluctance to report evidence of contamination and/or the use of research strategies that are unlikely to detect contamination, e.g., partial typing of samples, should not be interpreted as absence of contamination or as proof of authenticity of the data.

Experiments need to be redesigned with the goal of identifying all DNA contaminants, in order to differentiate convincingly between contamination and endogenous DNA. Richards et al. (1995) reported that approximately 50% of animal bones from an English site exhibited contamination with human sequences. Human bones should be assumed to be similarly contaminated. The inconsistent occurrence of the contaminating band in the samples presented here illustrates the insidious nature of very low level contamination and the need for constant vigilance in the detection of such contamination.

Ancient DNA or DNA contamination?

Several methods have been proposed as indicators for the suitability of specimens for aDNA studies. These protocols were intended to test the availability of amplifiable DNA, and we investigated their utility in detecting contamination as one dimension of a sample's suitability for aDNA study. Our results demonstrate that no analytical indicator, including protein analysis, amino acid racemization, DNA quantitation, or inability to amplify large DNA fragments, was able to predict the modern DNA contamination that was present in the studied samples. With amino acid quantitation, levels of amino acids were similar to those in fresh bone, suggesting that similar preservation of nucleic acids could be expected. With amino acid racemization, amino acids showed low levels of racemization, suggesting that amplifiable DNA might be present. However, the samples were contaminated with DNA, not protein, so that measures of protein quantity were unlikely to detect very low levels of DNA contamination. Therefore, a measure of DNA quantity was also determined. With DNA quantitation, between 10^3–10^6 amplifiable copies of PCR products ranging in size from 131–238 bp were present in the five DNA extracts. These values were equal to or greater than the minimum level of 10^3 copies suggested by Handt et al. (1996) for unambiguous, reproducible PCR results. Number of copies correlated negatively with PCR product size as expected for damaged, ancient DNA. However, the number of amplifiable copies was not markedly different in contaminated and uncontaminated samples. For example, samples 1 and 2 gave nearly identical quantitation results, yet sample 2 was contaminated and sample 1 was not. In sum, all of the methods gave positive indicators as to the amplifiability and antiquity of the DNA extracts, but none of them provided any indication of the contamination present in these samples.

Certain PCR controls have been proposed specifically to detect contamination, though they also proved inadequate to identify the current contamination. Negative extraction and PCR controls clearly missed the contamination. Similarly, the idea that undamaged DNA contaminants would outcompete damaged aDNA (Austin et al., 1997b) is probably an overly simplistic view of the manner in which nucleic acids interact. The two groups of DNAs did compete, but the results were not preordained and varied from sample to sample; increasing amounts of DNA extract resulted in increased amplification of the DNA contaminant in some cases and increased amplification of the endogenous DNA in other cases (data not shown). It has also been proposed that shorter PCR products are more likely to represent ancient DNA (Handt et al., 1994; Richards et al., 1995). However, the 238-bp PCR products from sample 2 were all ancient haplotype C, whereas the 132-bp products were a mixture of haplotype C and contaminating sequences.

How, then, does one determine which data constitute contamination and which reflect legitimately ancient DNA? The study by Stone and Stoneking (1998) of a prehistoric Amerind population from Illinois provides an excellent illustration of this dilemma. Out of the 25 DNA sequences that they determined, 22 could clearly be identified as one of the four New World haplogroups. The remaining three did not carry any of the

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sites diagnostic for New World haplogroups, although multiple extractions resulted in identical sequences. One sequence was excluded from analysis because it was identical to the haplotype of one of the authors, and one sequence was excluded because it matched one found in two Finnish individuals (Lahermo et al., 1996). However, the third sequence was retained because there was no compelling reason to exclude it. This sequence contains a 16278:T polymorphism that has been used to define haplogroup X, a novel New World founding haplotype proposed by Forster et al. (1996). Although Stone and Stoneking (1998) made no such claims, their data illustrate the problem in determining which data to accept as authentic in an aDNA study. Basically, does the burden of proof lie in providing support of the authenticity of the ancient sequence or in providing justification to exclude data? Given the problems illustrated in the current study, it seems prudent to require significantly more critical review of aDNA data than of modern DNA and to consider as preliminary any data that challenge historical orthodoxy. The data presented here can be used to illustrate the dangers of imprudent inclusion of data. The DNA sequence identified in sample 5 had never been detected in our laboratory or in New World indigenous populations. All associated extraction and PCR controls were negative. Multiple extractions resulted in the same RFLP/deletion haplotype. Therefore, it could be proposed that this haplotype represents a new founding lineage for the New World. However, the fact that this haplotype is found at high frequency in European populations (17%, Richards et al., 1996) and is not found in presumably ancestral Asian populations argues against this interpretation and against the inclusion of this sequence in a New World database.

In total, seven different non-New World sequences were identified in the current study. They are most likely all European in origin and may represent a minimum of seven independent sources of contamination. European populations are highly homogeneous from a mitochondrial perspective, and identification of a common European sequence such as the human reference sequence may conceal multiple sources of contamination. These results illustrate the problem in attempting to design a simple criterion by which to classify a sequence as endogenous. The most common sequence cannot be assumed to be endogenous, as illustrated by the predominant non-New World sequence in sample 4. Eliminating all author's sequences and previously reported non-New World sequences would still leave four out of seven contaminating sequences in the current study. In sum, there is no easy, objective method of identifying contaminating sequences other than to painstakingly analyze them within the genetic framework of the ancient population under study.

In the current study, the initial haplogroup assignments for each sample based on the RFLP/deletion analysis were ultimately unchanged by the DNA sequence analysis. However, this high level of accuracy was possible because of the presence of the large molecular database on New World indigenous populations that allowed simple identification of contaminating haplotypes. The situation would be more difficult for a different population or a less-studied locus or when dealing with an extinct lineage. Lacking an extensive database, only the thorough contamination protocol presented here would have revealed the extent of contamination present. For example, sample 5 could readily have been mistakenly identified as a novel founding lineage in the absence of a reference database or an extensive contamination investigation.

Ancient DNA applications to New World population studies

In theory, aDNA studies have the potential to make a significant contribution to our understanding of human population origins and evolution in the Americas and throughout the world. Since New World populations are relatively depauperate in mitochondrial variation, the identification of a novel founding lineage that has been lost in contemporary populations is a straightforward application of aDNA analyses. Many studies have also suggested that the morphology of Paleoindians differs from that of contemporary groups. A genetic analysis of the relevant archaeological specimens could provide an

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explicit answer as to whether or not these early populations became extinct or evolved into present-day populations. Thus far, the few data that exist on ancient Amerinds suggest that there is more plasticity in linguistic and osteological characters than [illegible] genetic change over time throughout the Americas and good continuity between ancient and contemporary populations. Finally, a genetic analysis of specimens from select geographic sites and temporal periods, corresponding to the independent waves of migration that have been proposed by Greenberg et al. (1986) and others, could provide a direct determination of the number of genetically distinct groups of people that migrated north out of Asia to colonize the New World. If the colonizing populations were independent, both geographically and temporally, their genetic distinctiveness should be most clearly manifest in prehistoric New World populations. Any one of these questions represents an interesting and worthwhile scientific investigation. The current reality, however, is that very few archaeological sites exist that contain well-preserved human remains likely to yield high-quality DNA and are from a geographic and temporal period that is of compelling relevance to questions of New World origins.

CONCLUSIONS

Each ancient DNA research project must be custom-designed with specific populations and polymorphic sites in mind. Polymorphisms that differentiate between ancient specimens and any potential sources of contamination must be identified and analyzed. This requirement is relatively simple to fulfill for New World populations due to the existence of polymorphic sites that distinguish them from European populations. However, the relative ease of contamination detection in New World populations is atypical of human aDNA studies, where ancient populations and likely source(s) of contamination are frequently genetically similar.

The incredible amounts of time and resources necessary for human aDNA studies mandate that only questions which cannot be addressed with modern samples be undertaken. The search for novel, extinct founding haplotypes is such an application. However, identification of a novel haplotype can make it difficult to fulfill the recommendation that polymorphisms be assayed which [illegible] discrimination between contaminating DNA and a haplotype that has never before [illegible]. In fact, the rarity of an aDNA sequence is often used as support of its authenticity. However, numerous novel sequences were recovered in the current study, yet it is unlikely that any of them represent new founding haplotypes. The impressive database of polymorphic DNA data in modern human populations assures the merit of a project assaying these markers in ancient populations, but only if extensive precautions have been taken to ensure the accuracy and reliability of the results.

A promising new application of aDNA technology is the analysis of ancient human pathogens, which can be conducted with a reduced risk of contamination. A recent study has detected DNA from *Treponema pallidum* subsp. *pallidum*, the causative agent for venereal syphilis, in a 200-year-old skeletal specimen from Easter Island (Kolman et al., in press). In this collaborative study, all work on modern treponemal DNA (including primer testing and PCR optimization) was conducted at the University of Washington, and all work on the archaeological specimen was conducted at the Smithsonian Institution. The geographic separation of modern and ancient DNA analyses and complete absence of modern treponemal specimens and DNA (including positive PCR controls) in the ancient DNA facility mean there is very little chance that the results reflect modern treponemal DNA contamination. A collaborative arrangement such as this, in which all research using modern DNA is conducted in one laboratory while the ancient specimens are analyzed in another laboratory, is critical for successful aDNA studies.

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APPENDIX. Complete D-loop DNA sequences of cloned amplification products

Human reference[1]	CAGCAATCAA 16211	CCCTCAACTA	TCACACATCA	ACTGCAACTC	CAAAGCCACC	CCTCACCCAC	TAGGATACCA	ACAAACCTAC	CCACCCTTAA 16300
Sample 1									
1.239[2]	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
2.239	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
4.239	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
6.239	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
Sample 2									
1.239	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
2.239	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
3.239	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
4.239	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
5.239	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
1.132	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
2.132	.....	..T.....	.....	.....	.....	.....	.....	.....	.....
4.132	.....G..	.....	.....	.....	.....	.....	.....	.....	.....
6.132	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
7.132	.....	..T.....	.....	.....	.....	T.....	.....	.....	.....C..
8.132	.....	..T.....	.....	.....	.....	.....	.....	.....	.....C..
9.132	.....	..T.....	.....	.....	.....	.....	...R.....	.....	.....C..
10.132	.....C...	..T.....	.....	.....	.....	.....	.....	.....	.....
Sample 3									
1.327	.....T..	.....	.....	.....	.....	.....	.....	.....	.....
2.327	.....T..	.....	.....	.....	.....	.....	.....	.....	.....
3.327	.....T..	...C.....	.....	.....	.....	.....	.....	.....	.....
4.327	.....C...	..T.....	.....	.....	.....	.....	.....	.....	.....
5.327	.....C...	..T.....	.....	.....	.....	.....	.....	.....	.....
6.327	.....C...	..T.....	.....	.....	.....	.....	.....	.....	.....
1.239	.....C...	.....	.....	.....	.....	.....	.....	.....	.....
2.239	.....C...	.....	.....	.....	.....	.....	.....	.....	.....
3.239	.....C...	.....	.....	.....	.....	.....	.....	.....	.....
4.239	.....	.....	.....	.....	.....	.....	.....	.....	T.....
5.239	.....	.....	.....T.	.....	.....	.....	.....	.....	.....
6.239	.....	.....	.....	.....	.....	.....	.....	.....	.....
7.239	.....	.....	.....	.....	.....	.....	.....	.....	.....
Sample 4									
1.327	.....	.....	.....	.....	.....	.....	.....	.....	...T.T....
2.327	.....	.....	.....	.....	.....	.....	.....	.....	...T.T....
3.327	.....C...	..T.....	.....	.....	.....	.....	.....	.....	.....
4.327	.....	.....	.....	.....	.....	.....	.....	.....	...T.T....
2.238	.....C...	.....	.....	.....	.....	.....	.....	.....	.....
3.238	.....	.....	.....	.....	.....	.....	.....	.....	...T.T....
4.238	.....	.....	.....	.....	.....	.....	.....	.....	.....
5.238	.....C...	.....	.....	.....	.....	.....	.....	.....	.....
Sample 5									
1.239	.....	.....	.....	.....	.....	.....	.....	.....	.....
2.239	.....	.....	.....	.....	.....	.....	.....	.....	.....
3.239	.....	.....	.....	.....	.....	.....	.....	.....	T.....
4.239	.....	.....	.....	.....	.....	.....	.....	.....	T.....
6.239	.....	.....	.....	.....	.....	.....	.....	.....	.....

(continued)

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APPENDIX (continued)

Reference	CAGTACATAG 16301	TACATAAAGC	CATTTACCGT	ACATAGCACA	TTACAGTCAA	ATCCCTTCTC	GTCCCCATGG	ATGACCCCCC	TCAGATAGGG	GTCCCTTGAC 16400
Sample 1										
1.239	. . . . .	. . . . .	. . . . C . T . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
2.239	. . . . .	. . . . .	. . . . C . T . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
4.239	. . . . .	. . . . .	. . . . C . T . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
6.239	. . . . .	. . . . .	. . . . C . T . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Sample 2										
1.239	. . . . .	. . . . .	. . . . C . T . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
2.239	. . . . .	. . . . .	. . . . C . T . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
3.239	. . . . .	. . . . .	. . . . C . T . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
4.239	. . . . .	. . . . .	. C . . C . T . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
5.239	. . . . .	. . . . .	. . . . C . T . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Sample 3										
1.327	. . . . .	. . . . .	. . . . . A . .	. . . . .	. . . . .	. . . . .	. C	. . . . .	. . . . .	. . . . .
2.327	. . . . .	. . . . .	. . . . . A . .	. . . . .	. . . . .	. . . . .	. C	. . . . .	. . . . .	. . . . .
3.327	. . . . .	. . . . .	. . . . . A . .	. . . . .	. . . . .	. . . . .	. C	. . . . .	. . . . .	. . . . .
4.327	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
5.327	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
6.327	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
1.239	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
2.239	. . A	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
3.329	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
4.239	. . . . . N	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
5.239	. . . . .	C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
6.239	. . . . .	C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
7.239	. . . . .	. . . . C . . G . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Sample 4										
1.327	. . . C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
2.327	. . . C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
3.327	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
4.327	. . . C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
2.238	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
3.238	. . . C	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
4.238	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
5.238	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Sample 5										
1.239	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
2.239	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
6.239	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .

[1] Mutations relative to the human reference sequence (Anderson et al., 1981) are depicted. Positions 16,211–16,300 are shown on the top half and positions 16,301–16,400 are shown on the bottom half of the appendix.

[2] Clones are labeled as number size of cloned PCR product in bp. Results for different samples are separated by a space, and results from independent amplification reactions are separated by a solid line.

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