Final Technical Report

NIJ Proposal to Enhance Methods for Studying Degraded DNA

Award Number: 2008-DN-BX-K008

Prepared by Brian M. Kemp, with contributions from Jodi Lynn Barta, Kelli Flanigan, Colin Grier, Cara Monroe, Justin E. Teisberg, and Misa Winters

ABSTRACT

Quantitative PCR was used to evaluate the efficacy of sodium hypochlorite in the removal of contaminating DNA from bone surfaces. While our findings are consistent with previous studies that found sodium hypochlorite to be highly efficient (~81-99%) at contamination removal, there emerged no treatment that removed 100% of the contamination across all of the experiments. Furthermore, this study suggests that previous claims that sodium hypochlorite is particularly damaging to endogenous ancient DNA (aDNA) molecules are inaccurate. Experiments conducted during this phase of the grant led to two additional relevant observations. First, mitochondrial DNA (mtDNA) preservation across individual bones was determined to be highly variable and not related to the density of the bone material, despite previous belief of such a relationship. Secondly, utilizing qPCR and a synthesized "standards" approach to measure the efficiency of some common DNA extraction methods for degraded skeletal samples, all methods were determined to perform poorly in retaining short segments of DNA. These findings challenge low copy number (LCN) expectations, suggesting that ancient and forensic specimens may contain far more preserved genetic material than previously recognized.

Secondly, we evaluated the overall effectiveness of nine different thermo-stable polymerases and polymerase blends in their ability to amplify mtDNA present in extractions from archaeological salmon vertebrae known to contain high levels of PCR inhibitors. Overall, Omni Klentaq LA outperformed the other 8 polymerases in two measures: 1) its success in permitting genetic species identification of these vertebrae, and 2) its ability to amplify an ancient DNA positive control when spiked with a volume of inhibited extract from the vertebrae.

Lastly, we evaluated the "behavior" and degree of post-mortem damage of DNA template molecules extracted from ancient human remains. Miscoding lesions observed in direct sequences correlated positively to amplicon length, which indirectly suggests that it is negatively related to starting template copy number. Thus, this is another characteristic of aDNA template molecules. Moreover, none of our PCRs were initiated from >1000 molecules, demonstrating that this cut-off, while regularly cited in the literature, is arbitrary for generating authentic aDNA results. Combined with results from previous studies, the degree of post-mortem damage appears to sample specific, making it difficult to generalize for all aDNA specimens and/or rely on a single set of recommendations as a means for absolute authentication. Overall, miscoding lesion damage was found to be random and, therefore, not to correlated to mutational hotspots, as previously argued for human mtDNA.

TABLE OF CONTENTS

EXECUTIVE SUMMARY

The analysis of DNA extracted from degraded human source materials is complicated by three major factors: 1) contaminating DNA, 2) co-extracted polymerase chain reaction (PCR) inhibitors, and 3) post mortem DNA damage. These associated problems make the authentication of DNA profiles from these sources particularly problematic, if not impossible. Common to both forensic science investigations and ancient DNA (aDNA) research, these issues have opened a fruitful dialogue between the fields (Alonso et al., 2004; Bär et al., 2000; Capelli et al., 2003; Kemp et al., 2006; Kemp and Smith, 2005; von Wurmb-Schwark et al., 2008). Despite recent advancements in both fields, there is a need for continued development of methods that increase the yield and purity of genetic material extracted from degraded sources. Alleviating these problems promises to facilitate future investigations of, for example, older cases (Nelson and Melton, 2007), cold cases (Isenberg, 2002; NIJ, 2002), war dead (Holland et al., 1993; Primorac et al., 1996), compromised samples (Foran, 2006), skeletal elements (Deng et al., 2005), missing persons (Bender et al., 2000), unknown victims (Erlich and Calloway, 2007), and mass fatality incidents (Prinz et al., 2007; Zehner, 2007).

This project was divided into three phases, one of each focused on contamination, PCR inhibition, or DNA damage. However, the results of the experiments from any one phase can and do address problems outside the major focus of the phase. The goal of this research was to validate methods that will improve reliability as well as reduce future time, cost, and effort needed for the analyses of degraded specimens. Additionally, these results have provided an increased understanding of the "behavior" of degraded DNA, knowledge that should continue to strengthen the legitimacy of this type of evidence.

Phase I: Contamination Removal from Ancient Specimens

As a part of the first phase of the grant, we evaluated the efficacy of sodium hypochlorite (i.e., Clorox bleach) to remove experimentally introduced contamination from the surfaces of bones, as well as determine its effect on DNA endogenous to the bones (these experiments are detailed in the main body of this report in the paper entitled "Further Evaluation of the Efficacy of Contamination Removal From Bone Surfaces"). Previously, both aDNA researchers and forensic scientists have conducted experiments aimed at evaluating methods of contamination removal, many of which conclude that it is difficult to entirely remove the contaminants (Gilbert et al., 2006; Malmstrom et al., 2005; Malmstrom et al., 2007; von Wurmb-Schwark et al., 2008). Moreover, there is concern that treatment with sodium hypochlorite will negatively impact the DNA endogenous to the sample. For example, Malmström and colleagues (2007) claimed that the amount of authentic aDNA is reduced by 77% when *powdered* bone or tooth is subjected to sodium hypochlorite before extraction.

Our experiments were conducted by bare hands handling ~3500 year old northern fur seal (*Callorhinus ursinus*) ribs to introduce contamination onto their surfaces. Subsections of the ribs were then removed from the whole and subjected to various concentrations of bleach. Following DNA extraction, quantitative PCR (qPCR) was employed to compare mitochondrial DNA (mtDNA) yields of both northern fur seal and human from treated bone sections to those left untreated.

Our findings are consistent with previous studies that found sodium hypochlorite to be highly efficient (~81-99%) at contamination removal, however there emerged no treatment that removed 100% of the contamination across all of the experiments. Moreover, the ability to estimate the degree of damage witnessed by endogenous northern fur seal molecules was compromised due to the inherent variability of preserved mtDNA across the bones, and the presence of co-extracted PCR inhibitors. These findings are in contrast to previous claims that sodium hypochlorite is particularly damaging to endogenous aDNA molecules and may in fact be highly inaccurate. We argue that assessing whether sodium hypochlorite "destroys" endogenous DNA is impossible, because the *exact same* sample of bone material would need to be assessed for endogenous DNA copy number *with and without* treatment. Nevertheless, in the pursuit of addressing this concern, additional observations were made that should positively impact standard forensic DNA practices.

While conducting the research just described, it became apparent to us that the inherent variability of mtDNA preserved across the northern fur seal ribs presented a serious obstacle to accurately evaluating the degree to which sodium hypochlorite negatively impacts DNA endogenous to the samples. In other words, it was observed that bleach treatment in some cases appeared to decrease the endogenous copy yield, whereas in other cases it appeared to increase the yields, which is impossible unless DNA is unevenly preserved across the bone section studied.

Data from these experiments, combined with those from additional qPCR experiments on the same collection of northern fur seal ribs further confirmed that indeed mtDNA preservation across a single bone is highly variable (these experiments are detailed in the main body of this report in the paper entitled "Mitochondrial DNA Preservation Across Individual Bones is Not Related to Bone Density"). The average preservation between the samples was significantly different (ANOVA, $p=1.9*10^{-9}$) with 15% of the total variance observed within samples. However, the majority the specimens (12 of 19, ~63.2%) exhibited at least an order of magnitude difference in mtDNA preservation across the whole.

Interestingly, while recent forensic research has focused on determining which skeletal elements are superior in their preservation of DNA over the long-term (e.g., Edson et al., 2009; Edson et al., 2004; Leney, 2006; Milos et al., 2007; Mundorff et al., 2009), less focus has been placed on measuring intra-element variation. Moreover, while there is a general belief that dense (cortical) bone material will contain better-preserved DNA than does spongy (cancellous) bone, to our knowledge this relationship has not been empirical demonstrated. To address this issue, we developed a novel measure taken on the rib subsections called the "density index", which amounts to an estimation of the density of a section of rib given its volume. Regression of the amount of mtDNA extracted per gram of the material against the density index of the bone from which it was extracted demonstrates no relationship between these variables ($R^2=0.03$, $p=0.28$). Similar experiments should be conducted on other skeletal elements to determine whether this lack of relationship is specific to ribs.

Another novel insight to emerge during this phase of the grant was the realization that, despite previous attempts to do so, different DNA extraction methods are not directly comparable in their recovery of low copy number (LCN) and degraded DNA. Previously, experiments have relied on the assumption that adding a relatively similar amount of sample (e.g., milligrams of bone) to each replicate at the beginning of an extraction,

allows the efficacy of the extraction to be tested by comparing the *relative* yields at the end of the protocol. Yet, because there was no knowledge of the *absolute* amount of DNA or recognition of the inherent variability of DNA preserved across single skeletal elements prior to extraction, the results from each method are *not directly comparable*. This idea became the foundation for another set of experiments conducted during this phase of the grant (these experiments are detailed in the main body of this report in the paper entitled "One of the Key Characteristics of Ancient and Forensic DNA, Low Copy Number, May be a Product of its Extraction").

DNA from ancient and forensic sources is generally believed to be of low copy number, despite the previous lack of direct measurement of DNA loss accumulated through the extraction process. We developed synthesized "standards" to measure the efficiency of some common DNA extraction methods for degraded skeletal samples, and used qPCR to estimate a known quantity of DNA subjected to a given extraction method (i.e., "copies in") versus quantity of DNA retained (i.e., "copies out"). All methods performed poorly in retaining short segments of DNA, giving low copy number results even when pre-extraction copy numbers far exceeded those expected of ancient samples. These findings challenge low copy number expectations, suggesting that ancient and forensic specimens may contain far more preserved genetic material than previously recognized. Furthermore, they emphasize the importance of optimizing and/or developing specialized extraction methods for retrieving degraded DNA.

Phase II: Co-Extraction of PCR Inhibitors and their Effects on Degraded DNA

Numerous substances can potentially inhibit PCR and are routinely encountered in both the study of aDNA (see review by Kemp et al., 2006) and forensic DNA (see review by Alaeddini, 2011). In these types of investigations, once contaminating DNA is sufficiently controlled for (e.g., see results from phase I of this report) and because the degree of post-mortem damage (Lindahl, 1993; Molak and Ho, 2011) witnessed by a sample *cannot* be controlled, co-purified PCR inhibitors remain as the greatest threat to the successful study of ancient, degraded, and/or LCN DNA samples.

The extent to which alternative forms of polymerases can tolerate inhibitory substances as well as amplify degraded samples is only beginning to be understood in aDNA and forensic studies. It is therefore compelling to determine which polymerase or manufactured blends of polymerases give the most optimal yield, fidelity, and resistance to a wide-range of inhibitors.

In this phase of the grant we evaluated the overall effectiveness of nine different thermo-stable polymerases and polymerase blends [1) Amplitaq Gold® DNA Polymerase (Invitrogen), 2) Herculase II Fusion DNA Polymerase (Agilent), 3) Omni Klentaq LA (DNA Polymerase Technology, Inc.), 4) Phusion® High Fidelity DNA Polymerase (Finnzymes), 5) Platinum® Taq DNA Polymerase (Invitrogen), 6) *Pwo* DNA Polymerase (Roche), 7) *rTth* (Applied Biosystems) 8) *Tfl* DNA Polymerase (Promega), and 9) $Vent_R$® DNA Polymerase (New England Biolabs)] in their ability to amplify mtDNA present in extractions from salmon vertebrae from two archaeological sites (DgRv-003 and DgRv-006) (these experiments are detailed in the main body of this report in the paper entitled "Evaluating the Efficacy of Various Thermo-Stable Polymerases Against Co-Extracted PCR Inhibitors in Ancient DNA Samples"). These samples were chosen for two reasons. First, in a previous study of salmon vertebrae from the DgRv-003 site (Grier et al., 2013), DNA extracted from the samples was found to be particularly challenging to

purify requiring on average 4.62 (SD= 2.31) repeat silica extractions to sufficiently remove the inhibitory effects (following Kemp et al., 2006). In our experience, these samples are some of the most inhibited samples we have ever worked with, rivaling even ~6000-9000 year old human fecal samples (or "coprolites") from archaeological sites located in Southeastern Utah (Kemp et al., 2010). Secondly, working with non-human, non-domestic animal samples minimizes the influence that contamination might otherwise have had.

In the end, we posed a simple question—that is, which of a number of commercially available polymerases or blends of polymerases performs best in the study of these highly inhibited samples. Our approach was not exhaustive to the number of polymerases available on the market; those tested represent only a portion of them. Performances were measured very simply as percent return of salmonid mtDNA sequences and percent of samples that amplified when spiked with an aDNA positive control. Overall, Omni Klentaq LA (DNA Polymerase Technology, Inc.) outperformed the other eight polymerases in these two measures. Use of this commercial polymerase is cost efficient and switching to this polymerase when working with samples that are highly compromised by PCR inhibitors requires no special training.

Phase III: Evaluating the Extent of Post Mortem DNA Damaged in Degraded Samples

The key to the success of any aDNA study relies on the authentication of results. While true of any line of scientific inquiry, conclusions drawn from the study of aDNA are only as strong as data generated to support them. Since DNA extracted from ancient remains is typically recovered in LCN and is short with regards to strand length (see review by Gilbert, 2006; Pääbo, 1989; Pääbo et al., 1988), the success of aDNA studies can be highly compromised by contamination originating from "modern" sources (Kemp and Smith, 2005; Malmstrom et al., 2005; Yang and Watt, 2005) and PCR inhibitors (Kemp et al., 2006). Moreover, ancient template molecules often exhibit further chemical modifications (e.g., miscoding lesions) that have occurred post-mortem (Gilbert et al., 2003a; Gilbert et al., 2003b; Hofreiter et al., 2001; Pääbo, 1989). If gone unrecognized, this form of damage can result in artificial "mutations" that can skew estimates of genetic diversity and mutation rates, and lead to problems in inferring demographic histories (Axelsson et al., 2008; Ho et al., 2007a; Ho et al., 2007b; Rambaut et al., 2009). The degree to which these problems will influence a study varies, with the study of ancient human DNA arguably being of the highest risk (Gilbert et al., 2005a).

Investigations of forensic and/or LCN DNA samples suffer similar problems of contamination, PCR inhibition, and post-mortem damage (Alaeddini, 2011; Alaeddini et al., 2010; Alonso et al., 2004; Bär et al., 2000; Capelli et al., 2003; Edson et al., 2004; Jobling and Gill, 2004; von Wurmb-Schwark et al., 2008). While over a decade ago, Fattorini and colleagues (2000) showed that reliable allele-specific oligonucleotide (ASO) probing could not be conducted on some forensic samples due to "artifacts" in the degraded DNA, today forensic DNA researchers and those faced with interpreting sequences from such aged and degraded specimens need a full appreciation for the extent of post mortem genetic damage. This topic has been approached by the forensic DNA community (Fattorini et al., 1999; Fattorini et al., 2000; Previdere et al., 2002), however recent discussion has been dominated by aDNA researchers (however, see the excellent review by Alaeddini et al., 2010). While artifactual mutations encountered in

aDNA studies might distort, for example, our reconstruction of the evolution of a species, their potential impact in forensic investigations is markedly more alarming. Thus, knowledge of the degree of damage exhibited by aged and/or degraded remains will guide interpretation, for example, of mtDNA mismatches to comparative samples, as has been needed to account for the markedly high mutation rate of the genome and prevalence of heteroplasmy (Buckleton et al., 2005; Tully et al., 2001).

In response to the recognized problems associated with the study of aDNA, a number of researchers have made recommendations to others in the field on how to properly conduct research and, in turn, authentic results (e.g., Cooper and Poinar, 2000; Gilbert et al., 2005a; Kemp and Smith, 2010; Pääbo et al., 2004; Willerslev and Cooper, 2005; Winters et al., 2011). While the views of these researchers vary, one point that appears to be widely agreed upon across the field is that aDNA should exhibit *appropriate molecular behavior*. On account of its degraded state, aDNA "behaves" differently than does "modern" DNA in experiments, an observation that stems from the seminal work of Pääbo and colleagues (1988) and Pääbo (1989) in which a strongly negative relationship between amplicon size and PCR efficiency was first noted.

Other recommendations for authenticating aDNA results rely on quantifying the number of template molecules that initiate a PCR. This recommendation is based on the idea that the number of staring template molecules should not be too large (which might indicate contamination) nor too small [which might permit miscoding lesions to be directly observed in the PCR product (see Figure 3 of Pääbo and colleagues (2004)]. There is also concern that when the starting number of aDNA molecules is very low subsequent PCRs will be particularly prone to contamination (Bunce et al., 2012; Cooper and Poinar, 2000), which at times can completely outcompete the aDNA during amplification (e.g., Kemp et al., 2006; Kemp and Smith, 2005).

In this phase of the grant, DNA extracted from 127 ancient human bone, teeth, and coprolite samples was amplified for eleven fragments varying in lengths from 62-1144 base pairs (bps) to evaluate the "behavior" and degree of post-mortem damage of aDNA template molecules (these experiments are detailed in the main body of this report in the paper entitled "Evaluating the Behavior and Degree of Post-Mortem Damage in Ancient DNA Template Molecules"). Of the 211 sequences not compromised by contamination, 34 (16.1%) exhibited miscoding lesions presumably due to post-mortem damage. Observation of this form of damage in direct sequences is positively related to amplicon length, which indirectly suggests that it is negatively related to starting template copy number. We have termed this phenomenon as "frequency dependent damage detection" (or FD^3), which is a characteristic of aDNA that may become useful in the authentication of future aDNA results. However, where qPCR results were generated in this study, they demonstrated no statistical difference in mean starting copy number for sequences that exhibited damage versus those that did not display damage ($p=0.25$ for 166 base pair (bp) amplicon, $p=0.486$ for 215 bp amplicon). Moreover, none of our PCRs were initiated from >1000 molecules, demonstrating that this cut-off, while regularly cited (stemming from the results described by Handt et al., 1996), is arbitrary for generating authentic aDNA results. Lastly, in this study we observed intact aDNA molecules in excess of 500 bps in length and found the contamination in this study to be both more degraded with regards to strand length and lower in copy number than some aDNA samples. Combined with results from previous studies, the degree of post-mortem damage appears to sample specific, making it difficult to generalize for all aDNA

specimens and/or rely on a single set of recommendations as a means for absolute authentication.

Due to the degree of miscoding lesions witnessed in aDNA molecules, many ancient DNA researchers have adopted cloning aDNA amplicons as opposed to directly sequencing them. However, no standardized protocol has emerged regarding the necessary number of clones to sequence, how a consensus sequence is most appropriately derived, or how results should be reported in the literature. In addition, there has been no systematic demonstration of the degree to which direct sequences are affected by damage or whether direct sequencing would provide disparate results from a consensus of clones.

To address this issue, during this phase of the grant we conducted a comparative study that examined both cloned and direct sequences recovered from ~3,500 year-old ancient northern fur seal DNA extracts (these experiments are detailed in the main body of this report in the paper entitled "To Clone or Not to Clone: Method Analysis for Retrieving Consensus Sequences in Ancient DNA Samples"). The goal of this study was to begin the systematic determination of whether a difference, if any, exists between cloning and direct sequencing in order to generate an aDNA consensus sequence. Working specifically with non-human, non-domesticate animal samples decreases the probability that contamination has contributed to these results

Majority rules and the Consensus Confidence Program (Bower et al., 2005) was used to generate consensus sequences for each individual from the cloned sequences, which exhibited damage at 31 of 139 base pairs across all clones. In no instance did the consensus of clones differ from the direct sequence. This study demonstrates that, when appropriate, cloning need not be the default method, but instead, should be used as a measure of authentication on a case-by-case basis, especially when this practice adds time and cost to studies where it may be superfluous.

Yet, it has been further argued that miscoding lesions occur non-randomly and "damage hotspots" may coincide with mutational hotspots. For example, in an experimental study of wheat (*Triticum aestivum*) DNA treated at 95°C for 2-21 days, Banerjee and Brown (2004) observed 107 of 124 cloned sequences to have damage at the same position in the atpA gene of the mitochondrial genome, resulting in an artifactual A>G transition. This, however, is in contrast to an earlier study of heat-treated wheat seeds, in which the damage appears to have been randomly distributed across the atpA gene and the A>G artifact just described was not observed (see figures 4 and 5 in Threadgold and Brown, 2003). Gilbert and colleagues (2003a) demonstrated that damage preferentially occurs at mutational hotspots in the mitochondrial hypervariable region I (HVRI) of the human mitochondrial genome, and that while coding region DNA (in this case the cytochrome oxidase III gene) damages less frequently, it shows no positional bias within codons. Gilbert and colleagues (2005b) also demonstrated that contamination did not likely influence this conclusion, as damage hotspots in ancient bison (*Bison bison*) mitochondrial control region DNA coincide with mutational hotspots. More recently, however, Kuch and colleagues (2007) found damage to be randomly distributed across HVRI, and only 1 of 10 damaged sites described by Dissing and colleagues (2008) is considered a mutational hotspot.

To address this outstanding issue and to follow-up on the cloning experiments conducted on the northern fur seal samples, in this portion of the third phase of the grant

we compared cloned and directly sequenced mitochondrial HVRI amplicons from ~220 to 6000 year old ancient human remains (these experiments are detailed in the main body of this report in the paper entitled “Cloning May Not Be a Necessary Criterion for the Authentication of Ancient DNA Consensus Sequences and Damage Appears to be Randomly Distributed Across the Human Mitochondrial HVRI Region”). As in the previous study of this phase of the grant, in no instance did the consensus of clones offer any added confirmation to the endogenous DNA sequence than did the direct sequence. From these same data, a relative rate of nucleotide damage was estimated across 255 cloned fragments and a comparison of expected to observed counts of substitutions was analyzed using a goodness of fit test. Post-mortem nucleotide damage across HVRI was shown not to deviate statistically from a random distribution ($G = 6.4992_{df=5}$, $P = 0.2606$). Moreover, there was not a strong relationship between damage hotspots and mutational hotspots, as previously argued for human mtDNA.

WORKS CITED

Alaeddini R (2011) Forensic implications of PCR inhibition--A review. Forensic Sci Int Genet 6:297-305.

Alaeddini R, Walsh SJ, and Abbas A (2010) Forensic implications of genetic analyses from degraded DNA--a review. Forensic Sci Int Genet 4:148-57.

Alonso A, Martin P, Albarran C, Garcia P, Garcia O, de Simon LF, Garcia-Hirschfeld J, Sancho M, de la Rua C, and Fernandez-Piqueras J (2004) Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. Forensic Science International 139:141-149.

Axelsson E, Willerslev E, Gilbert MT, and Nielsen R (2008) The effect of ancient DNA damage on inferences of demographic histories. Mol Biol Evol 25:2181-7.

Banerjee M, and Brown TA (2004) Non-random DNA damage resulting from heat treatment: implications for sequencing analysis of ancient DNA. Journal of Archaeological Science 31:59-63.

Bär W, Brinkmann B, Budowle B, Carracedo A, Gill P, Holland M, Lincoln PJ, Mayr W, Morling N, Olaisen B, Schneider PM, Tully G, and Wilson M (2000) Guidelines for mitochondrial DNA typing. Vox Sanguinis 79:121-125.

Bender K, Schneider PM, and Rittner C (2000) Application of mtDNA sequence analysis in forensic casework for the identification of human remains. Forensic Sci Int 113:103-107.

Bower MA, Spencer M, Matsumura S, Nisbet RE, and Howe CJ (2005) How many clones need to be sequenced from a single forensic or ancient DNA sample in order to determine a reliable consensus sequence? Nucleic Acids Res 33:2549-56.

Buckleton J, Walsh S, and Harbinson S (2005) Nonautosomal Forensic Markers. In J Buckleton, CM Triggs and S Walsh (eds.): Forensic DNA Evidence Interpretation. New York: CRC Press, pp. 299-340.

Bunce M, Oskam C, and Allentoft M (2012) Quantitative real-time PCR in aDNA research. In B Shapiro and M Hofreiter (eds.): Ancient DNA : Methods and Protocols. New York: Humana Press, pp. 121-132.

Capelli C, Tschentscher F, and Pascali VL (2003) "Ancient" protocols for the crime scene? Similarities and differences between forensic genetics and ancient DNA analysis. Forensic Science International 131:59-64.

Cooper A, and Poinar HN (2000) Ancient DNA: do it right or not at all. Science 289:1139.

Deng YJ, Li YZ, Yu XG, Li L, Wu DY, Zhou J, Man TY, Yang G, Yan JW, Cai DQ, Wang J, Yang HM, Li SB, and Yu J (2005) Preliminary DNA identification for the tsunami victims in Thailand. Genomics Proteomics Bioinformatics 3:143-57.

Dissing J, Kristinsdottir M, and Friis C (2008) On the elimination of extraneous DNA in fossil human teeth with hypochlorite. Journal of Archaeological Science doi:10.1016/j.jas.2007.10.009:1445-1452.

Edson SM, Christensen AF, Barritt SM, Meehan A, Leney MD, and Finelli LN (2009) Sampling of the Cranium of Mitochondrial DNA Analysis of Human Skeletal Remains. Forensic Sci Int 2:269-270.

Edson SM, Ross JP, Coble MD, Parsons TJ, and Barritt SM (2004) Naming the Dead-Confronting the Realities of Rapid Identification of Degraded Skeletal Remains. Forensic Science Review 16:64-90.

Erlich H, and Calloway CD (2007) Using HLA and Mitochondrial DNA Polymorphisms to Identify Geographic/Ethnic Origins: The Mammoth Lakes Case. Forensic Magazine.

Fattorini P, Ciofuli R, Cossutta F, Giulianini P, Edomi P, Furlanut M, and Previdere C (1999) Fidelity of polymerase chain reaction-direct sequencing analysis of damaged forensic samples. Electrophoresis 20:3349-57.
Fattorini P, Cossutta F, Giulianini P, Edomi P, and Previdere C (2000) DNA damage promotes mistyping in the allele specific oligonucleotide probing analysis of forensic samples. Electrophoresis 21:2969-72.
Foran DR (2006) Relative Degradation of Nuclear and Mitochondrial DNA: An Experimental Approach. J Forensic Sci 51:766-770.
Gilbert MT, Hansen AJ, Willerslev E, Rudbeck L, Barnes I, Lynnerup N, and Cooper A (2003a) Characterization of genetic miscoding lesions caused by postmortem damage. Am J Hum Genet 72:48-61.
Gilbert MT, Willerslev E, Hansen AJ, Barnes I, Rudbeck L, Lynnerup N, and Cooper A (2003b) Distribution patterns of postmortem damage in human mitochondrial DNA. Am J Hum Genet 72:32-47.
Gilbert MTP (2006) Postmortem Damage of Mitochondrial DNA. In H-J Bandelt, V Macaulay and M Richards (eds.): Human Mitochondrial DNA and the Evolution of Homo sapiens. Berlin: Springer-Verlag pp. 91-115.
Gilbert MTP, Bandelt H-J, Hofreiter M, and Barnes I (2005a) Assessing ancient DNA studies. TRENDS in Ecology and Evolution 20:541-544.
Gilbert MTP, Hansen AJ, Willerslev E, Turner-Walker G, and Collins M (2006) Insights into the Process behind the Contamination of Degraded Human Teeth and Bone Samples with Exogenous Sources of DNA. International Journal of Osteoaracheology 16:156-164.
Gilbert MTP, Shapiro B, Drummond AJ, and Cooper A (2005b) Post mortem DNA damage hotspots in Bison (Bison bison) provide evidence for both damage and mutational hotspots in human mitochondrial DNA. Journal of Archaeological Science 32:1053-1060.
Grier C, Flanigan K, Winters M, Jordan LG, Lukowski S, and Kemp BM (2013) Using Ancient DNA Identification and Osteometric Measures of Archaeological Pacific Salmon Vertebrae for Reconstructing Salmon Fisheries and Site Seasonality at Dionisio Point, British Columbia. Journal of Archaeological Science 40:544-555.
Handt O, Krings M, Ward RH, and Paabo S (1996) The retrieval of ancient human DNA sequences. American Journal of Human Genetics 59:368-376.
Ho SY, Heupink TH, Rambaut A, and Shapiro B (2007a) Bayesian estimation of sequence damage in ancient DNA. Mol Biol Evol 24:1416-22.
Ho SY, Kolokotronis SO, and Allaby RG (2007b) Elevated substitution rates estimated from ancient DNA sequences. Biol Lett 3:702-5.
Hofreiter M, Jaenicke V, Serre D, von Haeseler A, and Pääbo S (2001) DNA sequence from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. Nucleic Acids Research 29:4793-4799.
Holland MM, Fisher DL, Mitchell L, Rodriguez WC, Canick JJ, Merril CR, and Weedn VW (1993) Mitochondrial DNA sequence analysis of human skeletal remains: Identification of remains from the Vietnam War. Journal of Forensic Sciences 38:542-553.
Isenberg AR (2002) Forensic mitochondrial DNA analysis: A different crime-solving tool. The FBI Law Enforcement Bulletin.
Jobling MA, and Gill P (2004) Encoded evidence: DNA in forensic analysis. Nat Rev Genet 5:739-51.
Kemp BM, Monroe C, Geib PR, Matson RG, and Lipe WD (2010) Genetic Analysis of Coprolites from Southeastern Utah. 75th Annual Meeting of the Society for American Archaeology.

Kemp BM, Monroe C, and Smith DG (2006) Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts. Journal of Archaeological Science 33:1680-1689.
Kemp BM, and Smith DG (2005) Use of Bleach to Eliminate Contaminating DNA from the Surfaces of Bones and Teeth. Forensic Science International 154:53-61.
Kemp BM, and Smith DG (2010) Ancient DNA Methodology: "Thoughts from Brian M. Kemp and David Glenn Smith on "Mitochondrial DNA of Protohistoric Remains of an Arikara Population from South Dakota". Human Biology 82:227-238.
Kuch M, Grocke DR, Knyf MC, Gilbert MT, Younghusband B, Young T, Marshall I, Willerslev E, Stoneking M, and Poinar H (2007) A preliminary analysis of the DNA and diet of the extinct Beothuk: A systematic approach to ancient human DNA. Am J Phys Anthropol 132:594-604.
Leney MD (2006) Sampling Skeletal Remains for Ancient DNA (aDNA): A Measure of Success. Historical Archaeology 40:31-49.
Lindahl T (1993) Instability and decay of the primary structure of DNA. Nature 362:709-715.
Malmstrom H, Stora J, Dalen L, Holmlund G, and Gotherstrom A (2005) Extensive human DNA contamination in extracts from ancient dog bones and teeth. Mol Biol Evol 22:2040-7.
Malmstrom H, Svensson EM, Gilbert MT, Willerslev E, Gotherstrom A, and Holmlund G (2007) More on contamination: the use of asymmetric molecular behavior to identify authentic ancient human DNA. Mol Biol Evol 24:998-1004.
Milos A, Selmanovic A, Smajlovic L, Huel RL, Katzmarzyk C, Rizvic A, and Parsons TJ (2007) Success rates of nuclear short tandem repeat typing from different skeletal elements. Croat Med J 48:486-93.
Molak M, and Ho SY (2011) Evaluating the impact of post-mortem damage in ancient DNA: a theoretical approach. J Mol Evol 73:244-55.
Mundorff AZ, Bartelink EJ, and Mar-Cash E (2009) DNA preservation in skeletal elements from the World Trade Center disaster: recommendations for mass fatality management. J Forensic Sci 54:739-45.
Nelson K, and Melton T (2007) Forensic mitochondrial DNA analysis of 116 casework skeletal samples. J Forensic Sci 52:557-61.
NIJ (2002) Using DNA to Solve Cold Cases. National Institue of Justice.
Pääbo S (1989) Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proc Natl Acad Sci U S A 86:1939-43.
Pääbo S, Gifford JA, and Wilson AC (1988) Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Research 16:9775-9788.
Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, and Hofreiter M (2004) Genetic analyses from ancient DNA. Annu Rev Genet 38:645-79.
Previdere C, Micheletti P, Perossa R, Grignani P, and Fattorini P (2002) Molecular characterisation of the nucleic acids recovered from aged forensic samples. Int J Legal Med 116:334-339.
Primorac D, Andelinovic S, Definisgojanovic M, Drmic I, Rezic B, Baden MM, Kennedy MA, Schanfield MS, Skakel SB, and Lee HC (1996) Identification of War Victims from Mass Graves in Croatia, Bosnia, and Herzegovina by the Use of Standard Forensic Methods and DNA Typing. Journal of Forensic Sciences 41:891-894.
Prinz M, Carracedo A, Mayr WR, Morling N, Parson TJ, Sajantila A, Scheithauer R, Schmitter H, and Schneider PM (2007) DNA Commision of the International Society for Forensic Genetics (ISFG): Recommendations regarding the role of forensic genetics for disaster victim identification. Forensic Sci Int 1:3-12.

Rambaut A, Ho SY, Drummond AJ, and Shapiro B (2009) Accommodating the effect of ancient DNA damage on inferences of demographic histories. Mol Biol Evol 26:245-8.
Threadgold J, and Brown TA (2003) Degradation of DNA in artificially charred wheat seeds. Journal of Archaeological Science 30:1067-1076.
Tully G, Bar W, Brinkmann B, Carracedo A, Gill P, Morling N, Parson W, and Schneider PM (2001) Consideratons by the European DNA profiling (EDNAP) group on the working practices, nomeclature and interpretation of mitochondrial DNA profiles. Forensic Sci Int 124:83-91.
von Wurmb-Schwark N, Heinrich A, Freudenberg M, Gebuhr M, and Schwark T (2008) The impact of DNA contamination of bone samples in forensic case analysis and anthropological research. Legal Medicine 10:125-130.
Willerslev E, and Cooper A (2005) Ancient DNA. Proc Biol Sci 272:3-16.
Winters M, Barta JL, Monroe C, and Kemp BM (2011) To clone or not to clone: Method analysis for retrieving consensus sequences in ancient DNA samples. PLoS One 6:e21247.
Yang DY, and Watt K (2005) Contamination controls when preparing archaeological remains for ancient DNA analysis. Journal of Archaeological Science 32:331-336.
Zehner R (2007) "Foreign" DNA in tissue adherent to compact bone from tsunami victims. Forensic Science International: Genetics 1:218-222.

Further Evaluation of the Efficacy of Contamination Removal From Bone Surfaces

Jodi Lynn Barta[1,2,3], Cara Monroe[1,2,4], and Brian M. Kemp[1,2,*]

[1] School of Biological Sciences, Washington State University, Pullman, WA 99164
[2] Department of Anthropology, Washington State University, Pullman, WA 99164
[3] Department of Biological and Health Sciences, Madonna University, Livonia, MI 48150
[4] Department of Anthropology, University of California-Santa Barbara, Santa Barbara, CA 93106

*Corresponding Author:

Brian M. Kemp
Department of Anthropology
Washington State University
Pullman, WA 99164
Office: 509-335-7403
Fax: 509-335-3999
bmkemp@wsu.edu

Keywords: aDNA, low copy number (LCN) DNA, contamination, sodium hypochlorite, bleach, qPCR

ABSTRACT

Studies of low copy number (LCN) and degraded DNA are prone to contamination from exogenous DNA sources that in some cases can completely out-compete endogenous DNA in PCR amplification, thus leading to false positives and/or aberrant results. Particularly problematic is contamination that is inadvertently deposited on the surfaces of bones though direct handling. Whereas some previous studies have shown that contamination removal is possible by subjecting samples to sodium hypochlorite prior to DNA extraction, others caution that such treatment can destroy a majority of the molecules endogenous to the sample. To further explore this topic, we experimentally contaminated ancient northern fur seal (*Callorhinus ursinus*) ribs with human DNA and treated them with sodium hypochlorite to remove that contamination. Our findings are consistent with previous studies that found sodium hypochlorite to be highly efficient (~81-99%) at contamination removal, however there emerged no treatment capable of removing 100% of the contamination across all of the experiments. Moreover, the ability to estimate the degree of damage to endogenous northern fur seal molecules was compromised due to the inherent variability of preserved mtDNA across the bones, and the presence of co-extracted PCR inhibitors.

INTRODUCTION

The advent of the polymerase chain reaction (Saiki et al., 1985) revolutionized the fields of forensic and ancient DNA (aDNA) studies (e.g., Hagelberg et al., 1991; Hagelberg et al., 1989; Jeffreys et al., 1992; Pääbo, 1989; Pääbo et al., 1988). Subsequent advancements in PCR technology and chemistry have allowed DNA amplification from increasingly minute amounts of template molecules. While PCR now permits the routine study of genetic markers contained in degraded samples, it simultaneously represents a system that is hypersensitive to amplifying contaminant DNA (Kemp et al., 2006; Kwok and Higuchi, 1989; Yang et al., 2003).

Tempering the strength of DNA evidence collected from degraded remains and other aged biological materials is its challenging retrieval and authentication, principally because of the damage that the molecules have undergone since the death of the individual or the deposition of the biological material. Degradation by nucleases, oxidation, hydrolysis, deamination, and depurination lead to destabilization, breaks, and chemical modifications of DNA strands, leaving DNA template molecules that are few in number, typically short in length and carry “damaged” nucleotide positions (Gilbert et al., 2007; Gilbert, 2006; Hofreiter et al., 2001; Pääbo, 1989; Pääbo et al., 1988). As a result, studies of low copy number (LCN) and degraded DNA are prone to contamination from exogenous DNA sources that in some cases can completely out-compete endogenous DNA in PCR amplification, thus leading to false positives and/or aberrant results (e.g., Kemp and Smith, 2005).

There are four ways that contaminating DNA may be introduced into a study: 1) DNA introduced in the field (e.g., through handling), 2) DNA introduced by laboratory personnel, 3) cross contamination between samples and/or PCR products and samples, and 4) DNA present in pre-packaged laboratory reagents and/or present on labware. Addressing the second and third sources of contamination is generally an issue of maintaining high standards within the laboratory, so it is not surprising that forensic DNA researchers and others working with LCN and degraded DNA samples largely agree on a common set of practices (Butler, 2010; Cooper and Poinar, 2000; Kemp and Smith,

2010). Probably the most effective way to minimize contamination in the laboratory is to separate all pre- and post-PCR activities and equipment, especially when this is reinforced by rules that restrict movement from the post-PCR area back into the pre-PCR area before showering and changing into clean clothing. Additional safeguards that minimize contamination originating in the laboratory include wearing clean/disposable lab coats/suits, disposable gloves, hairnets, and facemasks. Purchasing goods and reagents guaranteed to be DNA-free can aid in minimizing the fourth source of contamination.

While educating those in the field about the potential threat they pose to the success of a study is the best line of defense against the first source of contamination (Edson et al., 2004; NIJ, 1999; Yang and Watt, 2005), contaminating DNA can be inadvertently deposited on the surfaces of bones and teeth through handling, from contact with other bodily fluids (e.g. perspiration or saliva), or when a specimen comes into contact with another contaminated object. In aDNA studies this source of contamination often enters the study during archaeological excavation and analyses (Yang and Watt, 2005). In the case of forensic investigation, crime labs are often presented with human remains that have poor provenience and have been directly handled (Steadman and Andersen, 2003). While Steadman and Anderson (2003) experimentally determined that latent fingerprints could be lifted from bones for the purpose of identifying those that have handled them, it is these very fingerprints that can compromise the success of a DNA investigation. While forensic researchers are most aware of the problems that contamination poses, law enforcement officers, morticians, and pathologists may be less informed (Baldwin and May, 2000; Mayo, 2004; Rutty, 2000; Rutty et al., 2000; Warrington, 2005). In order to spread awareness about DNA contamination throughout the law enforcement community, the National Institute of Justice published a pamphlet that encourages officers to “Wear gloves. Change them often”, “Use disposable instruments or clean them thoroughly before and after handling each sample”, “Avoid touching the area where you believe DNA to exist”, among other common sense suggestions (NIJ, 1999). One finds striking parallels with respect to aDNA studies where archaeologists are urged to “…change gloves and clean or change tools from one specimen to another…” (Yang and Watt, 2005: pg 334)

If contamination is detected, determining its source can be time consuming, expensive, and/or impossible when, for example, comparative DNA profiles of law enforcement officers and others involved with a case are nonexistent (Rutty, 2000). It is compelling, therefore, to develop a method that can remove contaminating DNA, while simultaneously having an insignificant effect on the endogenous target DNA. Achieving this goal could reduce the cost of future studies, make them less labor-intensive, and strengthen the weight of evidence gathered from degraded sources.

Subjecting bones and teeth to sodium hypochlorite (NaOCl or bleach) is one of the most common methods used for contamination removal (see studies reviewed by Kemp and Smith, 2005). Both aDNA researchers and forensic scientists have conducted experiments aimed at evaluating methods of contamination removal, many of which conclude that it is difficult to entirely remove the contaminants (Gilbert et al., 2006; Malmstrom et al., 2005; Malmstrom et al., 2007; von Wurmb-Schwark et al., 2008). It is most likely that failure to completely remove contamination stems from employing methods not robust enough to destroy the contaminants, as other studies have shown that complete decontamination is possible (Dissing et al., 2008; Kemp and Smith, 2005; Watt, 2005). For example, Kemp and Smith (2005) demonstrated that contaminating

human mitochondrial DNA (mtDNA), experimentally deposited on the surfaces of ancient human bones, could *only* be removed by submersion in ≥3.0% sodium hypochlorite[1] for 15 minutes. In contrast, studies by Malmström and colleagues (2005; 2007) and Gilbert and colleagues (2006) utilized 0.5-3.0% sodium hypochlorite which in turn failed to sufficiently remove contamination.

The ability to amply DNA from a bone or tooth after any treatment with sodium hypochlorite suggests that the endogenous DNA is protected from this heavy oxidant through its adsorption to hydroxyapatite, a bond not afforded to the contaminating DNA (Salamon et al., 2005). In fact, Kemp and Smith (2005) demonstrated that endogenous mtDNA was still recoverable from a bone fragment submerged in 6.0% sodium hypochlorite for twenty-one hours. In this case, it is likely that that the sodium hypochlorite had lost its activity at some point during treatment, but this was not considered by the authors. While mtDNA was recoverable following various other bleach treatments, Kemp and Smith (2005) did not evaluate the extent of degradation, if any, witnessed by the endogenous DNA during experimental treatments. Dissing and colleagues (2008) have shown that radioactively labeled hypochlorite (ClO^- containing the Cl^{36} isotope) migrates into the pulp of teeth after 30 minutes of submersion, suggesting, but not demonstrating, that a bleach treatment may indeed destroy some of the endogenous DNA that is well-preserved within the interior of a specimen. Utilizing quantitative PCR (qPCR), Malmström and colleagues (2007) argued that the amount of authentic aDNA is reduced by 77% when *powdered* bone or tooth is subjected to sodium hypochlorite before extraction.

To our knowledge, no investigations have evaluated the effect of bleach on endogenous DNA when whole bone fragments or teeth are treated for contamination. Here the efficacy of contamination removal from whole bone pieces by various treatments with bleach is measured in parallel to the effects that these treatments have on endogenous target DNA.

MATERIALS AND METHODS

Samples

The northern fur seal (*Callorhinus ursinus*) ribs studied here were excavated from the Amaknak Bridge Site in Unalaska, AK and date to approximately 3,500 years before present (YBP) (Crockford et al., 2004). They have an unknown handling history since excavation and have previously been determined to contain variable amounts of preserved mtDNA (Barta et al., in review; Winters et al., 2011). Segments of the ribs that remained after the study of Barta and colleagues (in review) were used in these experiments. These segments of bone span: 1) from the proximal pieces removed to the middle portions removed (here these remaining segments are called “proximal segments”) and 2) from the middle portions removed to the distal portion removed (here these remaining segments are called “distal segments”).

Experimental Contamination

[1] Many researchers inconsistently report their usage of bleach, leading to confusion about what has been employed (see discussion of this by Kemp and Smith, 2005). In this paper, all percentages of bleach represent percent of sodium hypochlorite (w/v). In this case 3% sodium hypochlorite is equivalent to a 1:1 dilution of full strength Clorox beach (6% sodium hypochlorite) to water.

Each segment was bare-hands handled by Kemp for twenty minutes, rotating the bone in his hand with the intention to thoroughly and evenly introduce contamination across the bone. Following this treatment, the segments were placed in a plastic bag, immediately given to Barta, and further processed within 24 hours. Cross-sections of bones were removed perpendicular to the length of ribs working in the direction from proximal to distal using a new Dremel® rotary blade for each cross-section removed. This work was conducted under a fume hood in the geoarchaeological laboratory at Washington State University, which is located in a separate building from the post-PCR facility. The inside surfaces of fume hood were wiped down with ~1.5% sodium hypochlorite before and after use. The first cross-section removed was intentionally not processed further. This was done because throughout the bare-hands handling procedure, the ends of the segments were also subjected to the introduction of contamination, so that the first cross-section might have been more contaminated because of its greater surface area exposed to contamination compared to the subsequent cross-sections removed. This was not an issue for the last cross-sections removed, as they did not include distal end of the segments.

Experimental Treatments

All subsequent preparation methods (i.e., experimental decontamination, DNA extraction and PCR set-up) were conducted in the aDNA laboratory at Washington State University, one dedicated to the analysis of degraded and low copy number (LCN) DNA. Appropriate measures to minimize contamination and, importantly, to detect it if present, were employed (Kemp and Smith, 2010).

Cross-sections removed from seven rib segments [809038 and 809039 proximal segments, and 809007, 809021, 809039, 809046 and 809053 distal segments (for the remainder of this paper these segments will simply be referred to as: 809007D, 809021D, 809038P, 809039P, 809039D, 809046D, and 809053D)] were examined in this study. Each portion of bone removed from the whole was weighed and cross section photographs were taken with an accompanying scale. The scaled photographs were imported into ImageJ 1.43s (Rasband, 1997-2012), and the total area of each cross section was recorded as the average of three measures taken (i.e., by tracing around the perimeter of the cross section). The thickness of each cross section was estimated by taking the average of 3-8 measures with digital calipers. From this, the density index of each cross-section was calculated following Barta and colleagues (in review) by dividing the volume (cross section area estimated from the photograph times the average thickness taken with calipers) of each cross-section by its weight (Table 1).

Cross-sections taken from across each rib segment were subsequently treated, prior to DNA extraction, as follows:

1. No treatment 1
2. Submersion in 30 mL of 6.0% sodium hypochlorite for 15 minutes
3. Submersion in 30 mL of 4.8% sodium hypochlorite for 15 minutes
4. Submersion in 30 mL of 3.6% sodium hypochlorite for 15 minutes
5. No treatment 2
6. Submersion in 30 mL of 3.0% sodium hypochlorite for 15 minutes
7. Submersion in 30 mL of 1.5% sodium hypochlorite for 15 minutes
8. Submersion in 30 mL of 0.6% sodium hypochlorite for 15 minutes

9. Submersion in 30 mL of water for 15 minutes [with the exception of sample 809021D]
10. No treatment 2

DNA Extraction and Quantification

DNA was extracted from the samples in batches, according to their segment, following Kemp and colleagues (2007). One or two extraction negative controls accompanied each extraction to monitor contamination.

Quantification by real time PCR was conducted with Applied Biosystems 7300 system with two different primer sets and three different probes. First, a 181 base pair (bp) portion of the cytochrome B gene of the northern fur seal mitochondrial genome was amplified and quantified following Winters and colleagues (2011).

Second, a 149 bp portion of the first hypervariable region (HVRI) of the human mitochondrial genome was amplified with primers 15986F and 16153R (Kemp et al., 2007) and quantified with two separate probes in two separate reactions. The first of these, designed to count all human DNA, was a MAR labeled probe 5'-GACTCACCCATCAACAACC-3' (Allelogic). This probe corresponds to nucleotide positions (nps) 16065-16083 of the Cambridge Reference Sequence (Anderson et al., 1981; Andrews et al., 1999). The second human probe was designed to count the amount of Kemp's mtDNA in each extract. Kemp is a member of mitochondrial subhaplogroup U2e and his mitochondrial genome exhibits a G>C transversion at nucleotide position (np) 16129. The locked nucleic acid (LNA) MAR labeled probe 5'-A+TATTG+TAC**C**GTACCA used in the Kemp assay corresponds to nps 16120-16135 [the bolded nucleotide reflects an intentional transversion from the Cambridge Reference Sequence (Anderson et al., 1981; Andrews et al., 1999) at np 16129. Cycling conditions included a 10 minute hold at 95°C followed by 50 cycles of 15 seconds at 95°C and 60 seconds at 55°C. At least four negative template controls were included on each 96-well plate to monitor contamination in reagents and ROX-labeled passive reference dye was included to correct for variation in well-to-well background fluorescence. Amplification curves were analyzed with the automatic baseline feature of the 7300 System SDS software (Applied Biosystems). Initial optimization investigations demonstrated that the Kemp probe was specific and could not be used to quantify non-Kemp human standards, but the human probe quantified both the Kemp standards and non-Kemp standards.

Mitochondrial DNA quantity was estimated from full concentration extracts from 2-3 reactions with each set of primers and respective probes. Similarly, mtDNA quantity was estimated from 1:10 dilutions of the extracts to explore the possible role of inhibitors in the data obtained from full concentration extracts. In all cases, quantifications that were "undetermined" by the qPCR system were recorded as zeros.

Data Analysis

The data collected for the northern fur seal mtDNA were used to calculate: 1) the average copy number per μL of extract (in the case of quantification from 1:10 dilutions these counts were multiplied by 10), 2) the average copy number per gram of material extracted [i.e. average copy number per μL times 100 (total extract volume) divided by the amount of bone processed], and 3) the standard deviation of those averages.

The Kemp and total human mtDNA quantification data were used to calculate: 1) the average copy number per μL of extract (in the case of quantification from 1:10 dilutions these counts were multiplied by 10), 2) the standard deviation of that average.

The percent inhibition (following the logic of Schwarz et al., 2009) between the full concentration extracts and their 1:10 dilutions was calculated as:

Inhibition (%)= 100 X [1-(average copy number per μL in the full extract/average copy number full per μL in the dilute extract)].

All statistical analyses were conducted in StatPlus with Microsoft Excel. An alpha value of 0.05 was used as the cut-off for statistical significance.

RESULTS AND DISCUSSION

Efficiencies of the northern fur seal DNA real time PCRs ranged from 90.62-97.32% as calculated from the slopes of the standard curves, with R^2-values ranging from 0.999-1.000. No northern fur seal mtDNA was detected in the extraction negative controls or the PCR negative reactions. Efficiencies for the human DNA real time PCRs that employed the Kemp probe ranged from 88.22-102.74% as calculated from the slopes of the standard curves, with R^2-values ranging from 0.998-0.999. No Kemp mtDNA was detected in the extraction negative controls or the PCR negative reactions. Efficiencies for the total human DNA real time PCRs that employed the human probe ranged from 84.34-106.20% as calculated from the slopes of the standard curves, with R^2-values ranging from 0.998-0.999. No human mtDNA was detected in the PCR negative reactions, but occasional low level human contamination was detected in some of the extraction negative controls, as discussed below.

Tremendous variation was observed in the qPCR measures taken in this study. It is first crucial to explore the degree of inhibition observed across the no treatment cross-sections. Twelve of the 21 (~57%) full concentration extraction cross-sections were demonstrably inhibited relative to the 1:10 dilute extraction, with percent inhibition ranging 6.11-48.46%. All three no treatment cross-sections from two segments, 809039D and 809053D, were found to be inhibited relative to their 1:10 dilutions. The average level of inhibition between all of the segments was not found to differ according to a one-way ANOVA (p=0.279), with 62% of the total variance being accounted for by intra-segmental variation. Given the inhibition effect observed in the no treatment cross-sections, all of the following results and discussion, where applicable, are based on the larger of the two counts of DNA quantification (i.e., from full concentration extracts or 1:10 dilution of those extracts) measured for the northern fur seal, Kemp, and human mtDNA (Table 1). This choice comes with the recognition that if the full concentration extract or its dilution is still inhibited, the average copy number counts for them will be underestimated.

A one-way ANOVA indicates that the average copy numbers per gram of the no treatment cross-sections are statistically different between the seven segments of bones (p=0.002), with 73% of the total variance accounting for inter-segment variation, and the remaining 27% accounting for within sample variation. This observation is consistent with statistically different average mean differences measured from proximal, middle, and distal portions of these same bones (one-way ANOVA, p=0.00004), where 88% of

the variance accounts for inter-segment variation [calculated here from the data presented by Barta and colleagues (in review)].

The average density index, measured from the no treatment cross-sections, is also statistically different between the seven segments of bones (one-way ANOVA, p=0.0002), with 81% of the total variance found between samples. This observation is inconsistent with the lack of difference detected in the average density, estimated from the same proximal, middle, and distal portions just mentioned (minus 809007D, for which these data were not available), of these bones (one-way ANOVA, p=0.196), where 42% of the variance accounts for inter-segment variation [calculated here from the data presented by Barta and colleagues (in review)]. Nevertheless, the lack of correlation between average copy number per gram and density index observed in this study (R^2=0.09, p=0.165), is consistent with the findings of Barta and colleagues (in review). This provides additional support for the observation that, despite a widely held belief, the density of cross sections of these bones is not predictive of DNA content (Barta et al., in review). It also demonstrates that the density of bone can vary tremendously along ribs.

The average amount of Kemp mtDNA, measured from the no treatment extracts, deposited on the seven segments of bones is statistically different (one-way ANOVA, p=0.00004), with 88% of the total variance accounted for by inter-segmental variation. Human mtDNA detected in these same extracts demonstrated a similar pattern, with average differences being statistically different (one-way ANOVA, p=0.00001) and 88% of the total variance accounted for by inter-segmental variation. Across five segments, the amount of Kemp mtDNA differed by an order of magnitude (809007D, 809039P, 809039D, 809046D, 809053D), two of which agree with the observation of human mtDNA (809007D, 809053D). A third segment also differed by an order of magnitude in the amount of human mtDNA deposited on it (809046D), but in a different direction than that of the Kemp mtDNA. Segment 809021D also had an order of magnitude of difference in the human mtDNA detected on it, but not for Kemp mtDNA. Lastly, segment 809038P demonstrated two orders of magnitude difference in both the Kemp and human mtDNA deposited across its surface. In total, variation in the Kemp mtDNA contamination observed between and within each segment was not anticipated, as the intensive and precise timing of the bare-hands handling of the bone was aimed to minimize just this variance. While remains typically studied in an aDNA or forensic context may not have been subject this level of handling, this is, nevertheless, quite illustrative to the fact that handling of remains can pose a threat spanning several orders of magnitude in its severity. Variation in the total human mtDNA contamination may have been further influenced by the handling history of these bones prior to them being delivered to the ancient DNA laboratory at Washington State University, the details of which are unknown to us.

With consideration of all of the variation described above, what lines of evidence can be drawn from the results of experimental attempts to remove contamination and assess the degree of damage witnessed by the endogenous DNA?

Regarding Kemp mtDNA contamination removal, for five of the seven segments results will be explored relative to the greatest amount of contamination observed on any one of the no treatment cross-sections. Since the most amount of Kemp mtDNA was detected in the water treated cross-section of 809039P and the 0.6% bleach treated cross-section of 809039D, these counts will be used as the baseline of contamination for the two segments, respectively. This approach will produce the most conservative estimate that

can be made of the efficacy of the treatments. Since complete contamination removal from the surface of a bone, if possible, should be the goal prior to DNA extraction, it is notable that this was achieved with: 1) 6.0% sodium hypochlorite treatment of 809007D, 809038P, and 809053D, 2) 4.8% sodium hypochlorite treatment of 809007D and 809038P, 3) 3.6% sodium hypochlorite treatment of 809007D, 809038P, and 809053D, 4) 3.0% sodium hypochlorite treatment 809007D, and 5) 1.5% sodium hypochlorite treatment 809007D (Table 2). It is the least surprising that contamination from the surfaces of 809007D cross-sections were easiest to remove, as the least amount of Kemp mtDNA was deposited across that segment (maximum 59.59 copies/μL, SD 28.48 copies/μL). Overall, there is a trend of increased contamination removal as the percent of sodium hypochlorite is increased from 0.6% (81.08% average removal, SD 36.32%) to 6.0% (99.78% average removal, SD 0.25%). However, it is notable that on average 46.29 % (SD 34.72%) of the contaminating molecules were removed simply by the submersion in water for 15 min.

The patterns of contaminating human mtDNA detected after experimental treatment mirrors that of the Kemp mtDNA observations (Table 1). However, it is notable that human mtDNA was detected in 11 of the 12 extraction negatives. The only extraction negative to not test positive for human mtDNA was the one that accompanied the extraction from segment 809053D (Table 1). No Kemp mtDNA was detected in any of the extraction negative controls. Since a low level of nonhuman, non-Kemp mtDNA entered the experiments after handling, it could not be removed by the experimental treatments tested here. Thus, these results are not applicable to the focus of this study.

Given the general effectiveness of sodium hypochlorite in the removal of contamination from the surface of bones [even at 1.5%, after 15 minutes 98.77% (SD 1.34%) of the Kemp contamination is estimated to have been removed], the next question to address is how much endogenous DNA has been destroyed during the process. Contrary to our expectation that this would have been straightforward to estimate with our experimental approach [as it has been done in the past on powered bones and teeth (Malmstrom et al., 2007)], the observations made in this study demonstrate that answering this question is very difficult and would probably be made with little accuracy. First, five of the seven segments produced experimental cross-sections that yielded copy numbers of northern fur seal mtDNA per gram that exceed yields from any of the respective no treatment cross-sections (Table 1): 1) water treatment of 809007D, 2) 3% sodium hypochlorite treatment of 809021D, 3) 3.6% and 3.0%, and 0.6% sodium hypochlorite treatments of 809038P, 4) water treatments of 809039P, and 5) 3.6% and 1.5% sodium hypochlorite treatments of 809039D. Moreover, in the case of three segments (809038P, 809039P, and 809039D) the 6.0% sodium hypochlorite treated cross-sections demonstrate higher copy numbers of northern fur seal mtDNA per gram than one of the no treatment cross-sections of the same bone. The copy number per gram of the 6.0% sodium hypochlorite treated cross-section of 809007D exceeded two no treatment cross-sections from that same bone. It is also notable that in no case did all of the sodium hypochlorite treated cross-sections yield lower northern fur seal mtDNA per gram than the lowest yield from a respective no treatment cross-section. Since our experimental treatments could only potentially destroy or remove endogenous DNA, these observations are probably the result of the inherent variability of mtDNA preserved across the bones [confirming the observations of Barta and colleagues (in review)]. It is possible also that some of the bleach treatments might have removed or subdued inhibitors (as suggested by Watt, 2005), thus permitting more accurate and, in this case, possibly larger copy number counts. Yet, taking this outcome into consideration requires further consideration of the

natural variance in inhibitors found across the bones. It is possible that some endogenous northern fur seal mtDNA was destroyed by any one of our sodium hypochlorite treatments, but the degree of that destruction is not anywhere as clear as the efficiency of bleach at removing contaminating exogenous mtDNA.

Given the results of the present study, it is instructive to compare them with previous studies. While Kemp and Smith (2005) found sodium hypochlorite to be effective at only 3.0% or higher for 15 minutes, they had no knowledge of the amount of contaminating molecules experimentally introduced in their study. Their observations were simply based on visible bands on polyacrylamide gels following PCR amplification, not from the number of molecules that initiated those reactions. While the patterns observed in this study are generally consistent with that of Kemp and Smith (2005), it is notable in this study how effective even low concentrations of bleach are on destroying exogenous contamination (Table 2).

While from this study, we are reluctant to calculate how much destruction to endogenous mtDNA is caused by sodium hypochlorite, Malmström and colleagues (2007) previously argued that the amount of authentic aDNA is reduced by 77% when *powdered* bone or tooth material is subjected to sodium hypochlorite before extraction. In this case, it is important to take a closer look at the methods and results of Malmström and colleagues (2007) to find possible discrepancies that might illustrate why the current data could not be used to draw a similarly confident claim. Malmström and colleagues (2007) compared the yields of dog mtDNA from powered bone and tooth material [data published by Malmström and colleagues (2005)] to that from powered tooth material from the same 23 individuals following submersion in 0.5% sodium hypochlorite for 15 minutes. However, in only seven of these cases was the copy number of the same 152 bp fragment directly compared. These data (reproduced here in Table 3) show that the amount of dog mtDNA from three sodium hypochlorite treated samples increased by 93.25-7048.57% over the no treatment comparisons. Copies of dog mtDNA from the other four sodium hypochlorite samples exhibited declines of 71.86-89.34% following sodium hypochlorite treatment. Nevertheless, on average over the seven samples, sodium hypochlorite treated samples yielded more dog mtDNA than those untreated (Table 3). It appears that Malmström and colleagues (2007) may have inadvertently detected the same inherent variability of endogenous copy number detected in this study (see also Barta et al., in review), but did not recognize it as such. However, Malmström and colleagues (2007) state that their estimate of a 77% decline of endogenous dog mtDNA derived from data presented in their Figure 2A. That figure depicts copy numbers of 111 bp fragments from across 25 samples and it is not clear how these data were compared to the 152 bp fragment from the 23 samples reported by Malmström and colleagues (2005). In any case, it is not appropriate to compare the copy number counts estimated by qPCR from two differently sized fragments (Pääbo et al., 2004) because of the negative relationship between fragment length and preserved copy number (Poinar et al., 2006; Schwarz et al., 2009). Furthermore, Malmström and colleagues (2007) did not control for endogenous copy number per unit of material extracted nor for potential complications due to the co-extraction of PCR inhibitors. In sum, this suggests that the average 77% loss figure reported by Malmström and colleagues (2007) lacks accuracy.

In addition, García-Garcerà and colleagues (2011) cautioned that sodium hypochlorite treatment of bones yields depurinated contaminant fragments, a characteristic that also occurs naturally post-mortem among the endogenous aDNA molecules. As such, they recommend against decontamination with sodium hypochlorite if shotgun sequencing is

to be employed. As illustrated by the data presented in this study, if bleach does not completely remove contamination, it can reduce it to levels lower than the endogenous aDNA (Table 1). In essence, contamination subjected to sodium hypochlorite can take on another characteristic of aDNA—low copy number. While this would not be a problem in the study of northern fur seal remains subjected to bleach to remove contaminating DNA, it does add complication to the study of forensic or ancient human remains treated for contamination prior to extraction. This possibility may have been easier to assess if García-Garcerà and colleagues (2011) had reported the percent sodium hypochlorite used to decontaminate their specimens[2]. Nonetheless, their observations are reminiscent of those made by Prince and Andrus (1992) who noted that very dilute sodium hypochlorite (0.1375%) caused nicks to DNA and thus, sodium hypochlorite treatment can cause contaminating DNA to take on characteristics of degraded aDNA.

CONCLUSIONS

One particularly problematic form of contamination is that which can inadvertently be deposited on the surfaces of bones though direct handling. Our findings are consistent with previous studies that found sodium hypochlorite to be highly efficient (~81-99%) at contamination removal, however there emerged no treatment that removed 100% of the contamination across all of the experiments. Moreover, the ability to estimate the degree of damage witnessed by endogenous northern fur seal molecules was compromised due to the inherent variability of preserved mtDNA across the bones, and the variable presence of co-extracted PCR inhibitors. The nature of these findings also suggest previous claims that sodium hypochlorite is particularly damaging to endogenous aDNA molecules may be highly inaccurate. Lastly, further exploration of contamination removal with sodium hypochlorite or alternative methods is needed given that this study reinforces sodium hypochlorite treatment can cause exogenous contaminating DNA molecules to take on characteristics of degraded and aDNA. If gone unrecognized, this can lead to false conclusions that results originating from contamination are indeed authentic.

ACKNOWLEDGMENTS

Thank you to Dr. Susan J. Crockford for turning our attention to the usefulness of ancient northern fur seals in these experiments. Thanks to Dr. Melissa Goodman Elgar for allowing us to use her laboratory for cutting the ribs. Special thanks to the Seattle Aquarium for providing modern northern fur seal samples to use for standards and positive controls. This project was supported by Award No. 2008-DN-BX-K008 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication/program/exhibition are those of the author(s) and do not necessarily reflect those of the Department of Justice.

[2] García-Garcerà and colleagues (2011) cite the decontamination employed by Ramirez and colleagues (2009), who report that bone powder was incubated in sodium hypochlorite for five minutes according Malmström and colleagues (2007). Since Malmström and colleagues (2007) used 0.5-3.0% sodium hypochlorite in their experiments, it is not clear exactly what was employed by García-Garcerà and colleagues (2011).

WORKS CITED

Anderson S, Bankier AT, Barrel BG, DeBulin MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, and Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457-465.

Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, and Howell N (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nature Genetics 23:147.

Baldwin HB, and May CP (2000) Crime Scene Contamination Issues. In JA Siegel, GC Knupfer and PJ Saukko (eds.): Encyclopedia of Forensic Sciences: Academic Press, pp. 412-416.

Barta JL, Monroe C, and Kemp BM (in review) Mitochondrial DNA Preservation Across Individual Bones is Not Related to Bone Density. Forensic Science International: Genetics.

Butler JM (2010) Fundamentals of Forensic DNA Typing: Elsevier.

Cooper A, and Poinar HN (2000) Ancient DNA: do it right or not at all. Science 289:1139.

Crockford SJ, Fredrick G, Wigen R, and McKechnie I (2004) Final Report on the analysis of the vertebrate fauna from Amaknak Bridge, Unalaska, AK, UNL050: Project # MGS-STP-BR-0310(S)/52930 to R. Knecht and R. Davis. Report on file, Alaska Department of Transportation, Anchorage.

Dissing J, Kristinsdottir M, and Friis C (2008) On the elimination of extraneous DNA in fossil human teeth with hypochlorite. Journal of Archaeological Science doi:10.1016/j.jas.2007.10.009:1445-1452.

Edson SM, Ross JP, Coble MD, Parsons TJ, and Barritt SM (2004) Naming the Dead-Confronting the Realities of Rapid Identification of Degraded Skeletal Remains. Forensic Science Review 16:64-90.

Garcia-Garcera M, Gigli E, Sanchez-Quinto F, Ramirez O, Calafell F, Civit S, and Lalueza-Fox C (2011) Fragmentation of contaminant and endogenous DNA in ancient samples determined by shotgun sequencing; prospects for human palaeogenomics. PLoS One 6:e24161.

Gilbert MT, Binladen J, Miller W, Wiuf C, Willerslev E, Poinar H, Carlson JE, Leebens-Mack JH, and Schuster SC (2007) Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis. Nucleic Acids Res 35:1-10.

Gilbert MTP (2006) Postmortem Damage of Mitochondrial DNA. In H-J Bandelt, V Macaulay and M Richards (eds.): Human Mitochondrial DNA and the Evolution of Homo sapiens. Berlin: Springer-Verlag pp. 91-115.

Gilbert MTP, Hansen AJ, Willerslev E, Turner-Walker G, and Collins M (2006) Insights into the Process behind the Contamination of Degraded Human Teeth and Bone Samples with Exogenous Sources of DNA. International Journal of Osteoaracheology 16:156-164.

Hagelberg E, Gray IC, and Jeffreys AJ (1991) Identification of the skeletal remains of a murder victim by DNA analysis. Nature 352:427-9.

Hagelberg E, Sykes B, and Hedges R (1989) Ancient Bone DNA Amplified. Nature 352:427-429.

Hofreiter M, Jaenicke V, Serre D, von Haeseler A, and Pääbo S (2001) DNA sequence from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. Nucleic Acids Research 29:4793-4799.

Jeffreys AJ, Allen MJ, Hagelberg E, and Sonnberg A (1992) Identification of the skeletal remains of Josef Mengele by DNA analysis. Forensic Sci Int 56:65-76.

Kemp BM, Malhi RS, McDonough J, Bolnick DA, Eshleman JA, Rickards O, Martinez-Labarga C, Johnson JR, Lorenz JG, Dixon EJ, Fifield TE, Heaton TH, Worl R, and Smith DG (2007) Genetic analysis of early Holocene skeletal remains from Alaska and its implication for the settlement of the Americas. American Journal of Physical Anthropology 132:605-621.
Kemp BM, Monroe C, and Smith DG (2006) Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts. Journal of Archaeological Science 33:1680-1689.
Kemp BM, and Smith DG (2005) Use of Bleach to Eliminate Contaminating DNA from the Surfaces of Bones and Teeth. Forensic Science International 154:53-61.
Kemp BM, and Smith DG (2010) Ancient DNA Methodology: "Thoughts from Brian M. Kemp and David Glenn Smith on "Mitochondrial DNA of Protohistoric Remains of an Arikara Population from South Dakota". Human Biology 82:227-238.
Kwok S, and Higuchi R (1989) Avoiding false positives with PCR. Nature 339:237-8.
Malmstrom H, Stora J, Dalen L, Holmlund G, and Gotherstrom A (2005) Extensive human DNA contamination in extracts from ancient dog bones and teeth. Mol Biol Evol 22:2040-7.
Malmstrom H, Svensson EM, Gilbert MT, Willerslev E, Gotherstrom A, and Holmlund G (2007) More on contamination: the use of asymmetric molecular behavior to identify authentic ancient human DNA. Mol Biol Evol 24:998-1004.
Mayo K (2004) Recovering Human Remains from Clandestine Graves. Evidence Technology Magazine 2:22-25.
NIJ (1999) What Every Law Enforcement Officer Should Know About DNA Evidence. National Institue of Justice.
Pääbo S (1989) Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proc Natl Acad Sci U S A 86:1939-43.
Pääbo S, Gifford JA, and Wilson AC (1988) Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Research 16:9775-9788.
Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, and Hofreiter M (2004) Genetic analyses from ancient DNA. Annu Rev Genet 38:645-79.
Poinar HN, Schwarz C, Qi J, Shapiro B, Macphee RD, Buigues B, Tikhonov A, Huson DH, Tomsho LP, Auch A, Rampp M, Miller W, and Schuster SC (2006) Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA. Science 311:392-4.
Prince AM, and Andrus L (1992) PCR How to Kill Unwanted DNA. Biotechniques 12:358-60.
Ramirez O, Gigli E, Bover P, Alcover JA, Bertranpetit J, Castresana J, and Lalueza-Fox C (2009) Paleogenomics in a temperate environment: shotgun sequencing from an extinct Mediterranean caprine. PLoS One 4:e5670.
Rasband WS (1997-2012) ImageJ. Bethesda, MD: National Institutes of Health.
Rutty GN (2000) Human DNA Contamination of Mortuaries: Does it Matter? Journal of Pathology 190:410-411.
Rutty GN, Watson S, and Davidson J (2000) DNA contamination of mortuary instruments and work surfaces: a significant problem in forensic practice? Int J Legal Med 114:56-60.
Saiki RK, Scharf SJ, Faloona F, Mullis KB, Horn GT, Erlich HA, and Arnheim N (1985) Enzymatic Amplification of B-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia. Science 230:1350-1354.

Salamon M, Tuross N, Arensburg B, and Weiner S (2005) Relatively well preserved DNA is present in the crystal aggregates of fossil bones. Proc Natl Acad Sci U S A 102:13783-13788.
Schwarz C, Debruyne R, Kuch M, McNally E, Schwarcz H, Aubrey AD, Bada J, and Poinar H (2009) New insights from old bones: DNA preservation and degradation in permafrost preserved mammoth remains. Nucleic Acids Res 37:3215-29.
Steadman DW, and Andersen SA (2003) Latent Print Processing of Human Bones. Journal of Forensic Identification 53:556-565.
von Wurmb-Schwark N, Heinrich A, Freudenberg M, Gebuhr M, and Schwark T (2008) The impact of DNA contamination of bone samples in forensic case analysis and anthropological research. Legal Medicine 10:125-130.
Warrington D (2005) Who Says You Can't Do That? Crime Scene Contamination. Forensic Magazine:31.
Watt KE (2005) Deconamination techniques in ancient DNA analysis. M.A. Thesis, Simon Fraser Univerisity, Burnaby, British Columbia.
Winters M, Barta JL, Monroe C, and Kemp BM (2011) To clone or not to clone: Method analysis for retrieving consensus sequences in ancient DNA samples. PLoS One 6:e21247.
Yang DY, Eng B, and Saunders SR (2003) Hypersensitive PCR, ancient human mtDNA, and contamination. Human Biology 75:355-364.
Yang DY, and Watt K (2005) Contamination controls when preparing archaeological remains for ancient DNA analysis. Journal of Archaeological Science 32:331-336.

Table 1. Results of northern fur seal, Kemp, and human mtDNA counted in this study across the seven segments of bones. The last three columns list the larger of the two counts recorded in the full concentration extraction or the 1:10 dilutions of those extracts. **For ease of viewing this table, it has been included as an excel file called "Appendix A" with this NIJ final report.**

Table 2. Maximum count of copy number per μL of Kemp mtDNA detected on the segments and average percent loss of Kemp mtDNA caused by the various treatments of contamination removal.

	Kemp Max copies/uL	6.0%	Percent Change	4.8%	Percent Change	3.6%	Percent Change	3.0%	Percent Change	1.5%	Percent Change	0.6%	Percent Change	Water	Percent Change
809007D	59.59	0.00	-100.00	0.00	-100.00	0.00	-100.00	0.00	-100.00	0.00	-100.00	11.13	-81.32	46.79	-21.48
809021D	269.66	0.66	-99.76	0.40	-99.85	0.85	-99.68	3.26	-98.79	0.82	-99.69	10.77	-96.01	n/a	n/a
809038P	165.78	0.00	-100.00	0.00	-100.00	0.00	-100.00	0.14	-99.91	0.19	-99.89	0.30	-99.82	107.49	-35.16
809039P	1534.08	5.41	-99.65	10.41	-99.32	11.09	-99.28	30.30	-98.03	57.40	-96.26	113.45	-92.60	1534.08	0.00
809039D	126.29	0.85	-99.33	0.47	-99.63	1.96	-98.45	0.48	-99.62	1.40	-98.89	126.29	0.00	3.76	-97.03
809046D	135.01	0.40	-99.70	0.75	-99.45	1.10	-99.19	1.26	-99.07	1.66	-98.77	2.45	-98.19	43.12	-68.06
809053D	121.99	0.00	-100.00	0.33	-99.73	0.00	-100.00	0.26	-99.79	2.59	-97.88	0.49	-99.60	53.63	-56.04
	Average Loss		**-99.78**		**-99.71**		**-99.51**		**-99.31**		**-98.77**		**-81.08**		**-46.29**
	SD		**0.25**		**0.26**		**0.58**		**0.72**		**1.34**		**36.32**		**34.72**

Table 3. Comparison of the results from Malmström and colleagues (2005) with those from Malmström and colleagues (2007). In the former study, the count of a 152 bp fragment of dog DNA was estimated from various samples, seven of which were replicated after treatment with 0.5% sodium hypochlorite for 15 minutes. The numbers in the column "average" was calculated from the average of the average counts from the first and second extraction. This count was compared to the average (or only count) from Malmström and colleagues (2007) to calculate the percent change of copy number after sodium hypochlorite treatment.

		Malmstrom and Colleagues (2005)			Malmstrom and colleagues (2007)	
Sample	**Element**	**First Extraction**	**Second Extraction**	**Average**	**Replication with sodium hypochlorite treament**	**% change**
2	bone	11 ± 15	52 ± 17	31.5	1680	5233.33
3	bone	24 ± 2	11 ± 1	17.5	1251 ± 1263	7048.57
4	bone	3867 ± 183	2097 ± 19	2982	839	-71.86
15	bone	2208 ± 365	5032 ± 366	3620	386	-89.34
16	bone	3251 ± 246	2996 ± 68	3123.5	744	-76.18
19	bone	4593 ± 651	4869 ± 596	4731	715	-84.89
22	tooth	694 ± 43	1411 ± 68	1052.5	2034	93.25
					Average % Change	**1721.84**
					SD	**3064.63**

Mitochondrial DNA Preservation Across Individual Bones is Not Related to Bone Density

Jodi Lynn Barta[1,2,3], Cara Monroe[1,2,4], and Brian M. Kemp[1,2,*]

[1] School of Biological Sciences, Washington State University, Pullman, WA 99164
[2] Department of Anthropology, Washington State University, Pullman, WA 99164
[3] Department of Biological and Health Sciences, Madonna University, Livonia, MI 48150
[4] Department of Anthropology, University of California-Santa Barbara, Santa Barbara, CA 93106

*Corresponding Author:

Brian M. Kemp
Department of Anthropology
Washington State University
Pullman, WA 99164
Office: 509-335-7403
Fax: 509-335-3999
bmkemp@wsu.edu

Keywords: Quantitative PCR, Ancient DNA, Bone Density

ABSTRACT

While recent forensic research has focused on determining which skeletal elements are superior in their preservation of DNA over the long term, little focus has been placed on measuring intra-element variation. Moreover, there is a general belief that dense (cortical) bone material will contain better-preserved DNA than does spongy (cancellous) bone. To address these ideas, quantitative PCR was used to estimate the degree of mitochondrial DNA (mtDNA) preservation variance across sections of 19 northern fur seal ribs (*Callorhinus ursinus*) that date to ~3500 years before present. Further, we developed a measure called the "density index" that was used to gauge the relative densities of the rib sections studied here to determine if density was an appropriate predictor of preservation. The average preservation between the samples was significantly different (ANOVA, $p=1.9*10^{-9}$) with only 15% of the total variance observed within samples. However, 12 of the 19 specimens (~63.2%) exhibited at least an order of magnitude difference in mtDNA preservation across the whole. Regression of the amount of mtDNA extracted per gram of bone material against the density index of the bone from which it was extracted demonstrates no relationship between these variables ($R^2=0.03$, $p=0.28$).

INTRODUCTION

It has been over two decades since DNA was first recovered from ancient bones (Hagelberg et al., 1989) and has since proved invaluable for forensic identification (Hagelberg et al., 1991; Jeffreys et al., 1992; Stoneking et al., 1991). Reflecting upon the first successful genetic identification of a murder victims' skeletal remains, Hagelberg and colleagues (1991: pg 429) noted that, "First, it is unclear what proportion of skeletal remains will yield sufficient human DNA…" and, "Second, in the case of an apparent exclusion based on bone DNA analysis, it may be impossible to determine whether the exclusion is authentic or is inadvertently derived from contaminating material." These possible drawbacks stem from the fact that DNA preserved in ancient and degraded remains are generally recovered in low copy number and are short with regards to strand length (Gilbert, 2006; Pääbo, 1989; Pääbo et al., 1988). Molecules may also exhibit some additional chemical modifications (i.e., "miscoding lesions") (Gilbert et al., 2003a; Gilbert et al., 2003b; Hofreiter et al., 2001; Pääbo, 1989). Consequently, retrieval of ancient and degraded DNA is challenging, and attempts to authenticate human data can be compromised both by contamination, and by miscoding lesions that can appear as "mutations", when in fact they are artifacts. It is interesting to note that, as these challenges were encountered in forensic and ancient DNA (aDNA) investigations, remarkably similar protocols for minimizing contamination (NIJ, 1999; Yang and Watt, 2005) were developed for field workers in the respective disciplines (e.g. local law enforcement and archaeologists) .

Following Hagelberg and colleagues' (1991) assertion that not all skeletal remains may contain DNA, forensic research has sought to determine which skeletal elements are superior in their preservation of DNA, regardless of postmortem interval, depositional and taphonomic history, and/or difficulty associated with processing of the material (Edson et al., 2009; Edson et al., 2004; Leney, 2006; Milos et al., 2007; Mundorff et al., 2009). Despite forensic genetic analyses of over 31,000 skeletal elements that date as early as AD 1941 (summarized in Table 1), the determination of which skeletal element is most ideal for genetic analysis remains elusive. In general, the rank order of successful skeletal elements varies study by study. Nevertheless, it has been argued,

from subsets of the data presented in Table 1, that sampling of dense cortical bone from weight bearing elements (e.g., femora, tibiae, and metarsals) is most beneficial (Edson et al., 2004; Leney, 2006; Milos et al., 2007). The notion that the density of a bone is positively correlated with DNA preservation seems to be so highly recognized that sampling dense, weight bearing bone over less dense material has become a general recommendation for DNA studies (NIJ, 2005; Parsons and Weedn, 1997; Prinz et al., 2007). However, this preference is inconsistent with a number of observations. For example, it is difficult to reconcile the high success of rib samples (96.15%) reported by Edson and colleagues (2004) over that for femora, tibiae, and metatarsals (80.95-94.79%)(Table 1). To further confound this issue, Milos and colleagues (2007: pg 492) avoided sampling ribs altogether because of their "…initial experience that they are an inferior source of DNA". Thus, no data for ribs are available from the largest study to date that aimed to evaluate differential DNA preservation across skeletal elements, even though a reasonably low overall success rateof 22.75% for ulnae, for example, was reported in the study (Milos et al., 2007) (Table 1). Moreover, Misner and colleagues (2009) found no statistical difference in the success rate or DNA quantity between femora and ribs. These observations suggest that density alone may not explain the difference in success when sampling ribs or weight bearing elements.

Most studies found cranial fragments to rank low, with on overall position of 24th (of 25) across all classes of elements (summarized in Table 1). However, when results from cranial fragments are sorted by specific cranial element, successful analysis of DNA from temporal bones (90%) rivals that of weight bearing elements (Edson et al., 2009). This success is thought to be a product of sampling the relatively dense petrous portion of the temporal bone (based on unpublished data by Misner et al., 2009). Therefore, in this case, it appears that the density of a bone may be more critical to DNA preservation than the role of the skeletal element in supporting weight. DNA extracted from other non-weight bearing dense bones, such as the mandible and the patella, has been met with relatively high success (Table 1) and this has been attributed to non-weight bearing, functional stresses demanded by these bones (Leney, 2006; Mundorff et al., 2009).

Previous studies which aimed at determining the relationship between DNA preservation and bone density are problematic because there has been little information reported about how the relative density of bones was ascertained. For example, most recognize that the midshaft of the femur is very dense, but how dense is it relative to the humerus? More importantly, how is the relative density of a particular element related to its relative success rate? In one case the relative rank order of fibula density among other elements (femur > tibia > humerus > radius > ulna > fibula) did not relate to its relative rank order of successful analysis among these same elements (femur > fibula > tibia > humerus > radius > ulna) (Milos et al., 2007). One major drawback to the data presented in Table 1 is that none of the studies are based on actual quantification of DNA. In addition, as the relative amount of each bone processed was not reported the relative amount of DNA yield per unit of bone extracted is not possible to calculate. Without such information about the relative density of a bone and its relative DNA yield, the relationship between these two characteristics remains crudely understood. Thus, it may be premature to generalize about which skeletal element(s) are superior for genetic analysis.

Lastly, it remains unknown how variable DNA preservation is across single skeletal elements. In other words, is there not only a best skeletal element to choose for genetic analysis and also a best place to sample from within that skeletal element? It has been

demonstrated, for example, that the degree of post-mortem degradation and DNA copy number is not uniform across the mineral and organic components of bone (Salamon et al., 2005; Schwarz et al., 2009). It is currently unknown whether this may also apply to DNA preservation. The purpose of this study is to measure the variation in DNA preservation across individual ancient northern fur seal (*Callorhinus ursinus*) ribs in order to test the relationship between relative DNA preservation and bone density.

MATERIALS AND METHODS

Samples

The 19 northern fur seal ribs studied here were excavated from the Amaknak Bridge Site in Unalaska, AK and date to approximately 3,500 years before present (YBP) (Crockford et al., 2004). The work in this study was performed with non-human, non-domestic animal remains in order to minimize the potential influence of contamination on the results.

Calculation of the Density Index

Cross-sections of bone were removed perpendicular to the length of ribs from the proximal, middle, and distal portions using a new Dremel® rotary blade for each piece removed (Table 2). This work was conducted under a fume hood in the geoarchaeological laboratory at Washington State University, a lab located in a separate building from both the pre-PCR and the post-PCR facilities. Before and after use of the fume hood, its surfaces were wiped down with ~1.5% sodium hypochlorite (25% dilution of Clorox bleach). Each portion of bone removed from the whole was weighed and cross section photographs were taken of the portions removed from 16 of the samples with an accompanying scale (minus the image of the distal piece of 809020, which was inadvertently lost). The scaled photographs were imported into ImageJ 1.43s (Rasband, 1997-2012), and the total area of each cross section was recorded as the average of three measures taken (i.e., by tracing around the perimeter of the cross section). The thickness of each cross section was estimated by taking the average of 3-8 measures with digital calipers.

To compare the relative density of each bone section processed, a measure was created that is here referred to as the “density index”. This was calculated by dividing the volume (cross section area estimated from the photograph times the average thickness taken with calipers) of each sample by its weight. This method of measuring density is aimed at minimizing assumptions about the shape of the bone, for example no bone is a perfect cylindrical shape (Galloway et al., 1997).

DNA Methods

All preparation methods (i.e., extraction and PCR set-up) were conducted in the aDNA laboratory at Washington State University, one dedicated to the analysis of degraded and low copy number (LCN) DNA. Appropriate measures to minimize contamination and, importantly, to detect it if present, were employed (Kemp and Smith, 2010).

DNA Extraction and Quantification

DNA was extracted from the samples in batches of three sections (proximal, middle, and distal), with an accompanying extraction negative control, following Kemp and colleagues (2007), minus the bleach decontamination step.

A 181 base pair (bp) portion of the cytochrome B gene of the mitochondrial genome was amplified and quantified by real time PCR following Winters and colleagues (2011). In addition to the quantification of full concentration extracts, quantification of 1:10 and 1:20 dilutions of those extracts was conducted to explore the possible role of inhibitors in the data obtained from full concentration extracts. In all cases, quantifications that were "undetermined" by the qPCR system were recorded as zeros.

Data Analysis

These data were used to calculate the following: 1) the average copy number per μL of extract (in the case of the 1:10 and 1:20 dilutions, these counts were multiplied by 10 and 20 respectively), 2) the standard deviation of that average (if more than one measure was taken), 3) and the average copy number per gram of material extracted [i.e. average copy number per μL times 100 (total extract volume) divided by the amount of bone processed]. The percent inhibition (following the logic of Schwarz et al., 2009) between the full concentration extracts and their dilutions was calculated as:

Inhibition (%) = 100 X [1-(average copy number per μL in the full extract/average copy number full per μL in the dilute extract)].

All statistical analyses were conducted in StatPlus with Microsoft Excel. An alpha value of 0.05 was used as the cut-off for statistical significance.

RESULTS AND DISCUSSION

Efficiencies of the real time PCRs ranged from 85.73-96.16% as calculated from the slopes of the standard curves, with R^2-values ranging from 0.997-1.000. No northern fur seal mtDNA was detected in the extraction negative controls or the PCR negative reactions.

The 57 extractions from the 19 ribs exhibit a tremendous amount of both intra- and inter-sample variation regarding inhibition effects (Table 2). In comparison to the 1:10 dilute extracts, only 10 of 57 full concentration extracts (809003D, 809011M, 809012M, 809034P, 809038D, 809044M, 809044M, 809044D, 809046D, and 809053D) demonstrated no inhibition (i.e., they demonstrate a negative percent inhibition, Table 2). Three of these samples (809003D, 809011M, and 809012M) were inhibited relative to their 1:20 dilute extracts (i.e., they demonstrate a positive percent inhibition). This suggests the 1:10 dilutions were insufficient to overcome the inhibitor effect for these samples. The other 47 full concentration extracts were calculated to be inhibited ranging from 3.13-100%. On the highest end, sample 809020D was not quantifiable at full concentration, but a 1:10 dilution of this extract demonstrates ~301.56 copies/μL. One sample that was strongly inhibited as a result of co-extracted PCR inhibitors, was 809039P that exhibited 71.5 copies/μL (SD 119.08) [quantified three separate times, the extract was counted as 1.06, 4.46, and 209 copies/μL] in the full, but the 1:10 dilution was quantified at 4302.71 copies/μL (SD 563.36). One-way ANOVA demonstrates that

the average level of inhibition observed in 19 ribs does not differ between samples (p=0.32) and that 64% of the total variance is found within samples, with the remaining 36% accounting for between sample variation.

In comparison to the 1:20 dilute extracts, only 8 full concentration extracts (809005P, 809026D, 809034P, 809034D, 809044M, 809048M, 809046D, and 809053D) demonstrated no inhibition (Table 2). The other 49 full concentration extracts were calculated to be inhibited ranging from 0.6-99.74%.

The northern fur seal rib samples also exhibited a tremendous amount of both intra- and inter-sample variation regarding DNA preservation, estimated as the average copy number count of a 181 bp fragment of the mitochondrial cytochrome B gene per gram of bone extracted. To compensate for the complications arising from the presence of co-extracted PCR inhibitors in these extracts, the following results were derived from taking the maximal count from across the full concentration extract and dilutions as the best estimation of average copy number count per gram for each bone section studied (Table 2). Note that if any extract or dilution is still inhibited, the average copy number counts for them will be underestimated. One-way ANOVA demonstrates that the average level of mtDNA preservation is statistically significant among the 19 ribs ($p=1.9*10^{-9}$). Tremendous inter-sample variance in DNA preservation is not unexpected in the study of ancient specimens (e.g., Malmstrom et al., 2005; Schwarz et al., 2009). Overall 15% of the total variance is found within samples, with the remaining 85% accounting for between sample variance. While overall most average copy variance is between samples, it is important to highlight that: 1) eight bones (809003, 809005, 809009, 809012, 809026, 809039, 809044) reveal one order of magnitude difference in mtDNA preservation across the bone, 2) three bones (20035, 809038, 809048) two orders of magnitude difference, and 3) DNA preservation across bone 809045 varies by three orders of magnitude. Thirteen of the rib samples exhibited the highest level of mtDNA preservation in the proximal end, five in the distal end, and a single specimen in the middle.

The density indices measured in this study ranged from 0.49-1.83 (Table 2). This indicates, for example that 809012D is ~3.7 times less dense than 809048D (Figure 1). One-way ANOVA demonstrates that the average DI measured in 19 ribs does not differ between samples (p=0.55) and that 69% of the total density variance is found within samples, with the remaining 31% accounting for between sample variation.
A regression of the density index against the average copy number/g demonstrates that there is no relationship between these two variables ($R^2=0.03$, p=0.28) (Figure 2).

CONCLUSION

In the pursuit of determining which skeletal elements are superior in their preservation of DNA over the long term, little attention has been focused on the degree of intra-elemental variation. This study of 3500-year-old northern fur seal ribs, found that only 15% of the total variance in mtDNA preservation was accounted for by within sample variance. However, the majority of the specimens exhibited at least an order of magnitude difference in mtDNA preservation across the whole bone. This suggests that those investigating inter-elemental variation should also consider variation within elements, and make efforts to relate DNA yield to the amount of material processed as we have done here. Furthermore, across all 57 extractions, no relationship between the density of the bone and its level of mtDNA preservation was found, despite a general

belief by researchers that a positive relationship exists. Similar experiments should be conducted on other skeletal elements to determine whether this lack of relationship is specific to ribs. We note that is may be difficult if the bones tested are of variable levels of dryness, as moisture can influence the density index independent of the fraction of which a bone is composed of cortical or cancellous bone.

ACKNOWLEDGMENTS

Thank you to Dr. Susan J. Crockford for turning our attention to the usefulness of ancient northern fur seals in these experiments. Thanks to Dr. Melissa Goodman Elgar for allowing us to use her laboratory for cutting the ribs. Special thanks to the Seattle Aquarium for providing modern northern fur seal samples to use for standards and positive controls. This project was supported by Award No. 2008-DN-BX-K008 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication/program/exhibition are those of the author(s) and do not necessarily reflect those of the Department of Justice.

WORKS CITED

Crockford SJ, Fredrick G, Wigen R, and McKechnie I (2004) Final Report on the analysis of the vertebrate fauna from Amaknak Bridge, Unalaska, AK, UNL050: Project # MGS-STP-BR-0310(S)/52930 to R. Knecht and R. Davis. Report on file, Alaska Department of Transportation, Anchorage.

Edson SM, Christensen AF, Barritt SM, Meehan A, Leney MD, and Finelli LN (2009) Sampling of the Cranium of Mitochondrial DNA Analysis of Human Skeletal Remains. Forensic Sci Int 2:269-270.

Edson SM, Ross JP, Coble MD, Parsons TJ, and Barritt SM (2004) Naming the Dead-Confronting the Realities of Rapid Identification of Degraded Skeletal Remains. Forensic Science Review 16:64-90.

Galloway A, Willey P, and Snyder L (1997) Human Bone Mineral Densities and Survival of Bone Elements: A Contemporary Sample. In WD Haglund and MH Sorg (eds.): Forensic Taphonomy: The Postmortem Fate of Human Remains. Boca Raton, Florida: CRC Press, pp. 295-317.

Gilbert MT, Hansen AJ, Willerslev E, Rudbeck L, Barnes I, Lynnerup N, and Cooper A (2003a) Characterization of genetic miscoding lesions caused by postmortem damage. Am J Hum Genet 72:48-61.

Gilbert MT, Willerslev E, Hansen AJ, Barnes I, Rudbeck L, Lynnerup N, and Cooper A (2003b) Distribution patterns of postmortem damage in human mitochondrial DNA. Am J Hum Genet 72:32-47.

Gilbert MTP (2006) Postmortem Damage of Mitochondrial DNA. In H-J Bandelt, V Macaulay and M Richards (eds.): Human Mitochondrial DNA and the Evolution of Homo sapiens. Berlin: Springer-Verlag pp. 91-115.

Hagelberg E, Gray IC, and Jeffreys AJ (1991) Identification of the skeletal remains of a murder victim by DNA analysis. Nature 352:427-9.

Hagelberg E, Sykes B, and Hedges R (1989) Ancient Bone DNA Amplified. Nature 352:427-429.

Hofreiter M, Jaenicke V, Serre D, von Haeseler A, and Pääbo S (2001) DNA sequence from mulitple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. Nucleic Acids Research 29:4793-4799.

Jeffreys AJ, Allen MJ, Hagelberg E, and Sonnberg A (1992) Identification of the skeletal remains of Josef Mengele by DNA analysis. Forensic Sci Int 56:65-76.

Kemp BM, Malhi RS, McDonough J, Bolnick DA, Eshleman JA, Rickards O, Martinez-Labarga C, Johnson JR, Lorenz JG, Dixon EJ, Fifield TE, Heaton TH, Worl R, and Smith DG (2007) Genetic analysis of early Holocene skeletal remains from Alaska and its implication for the settlement of the Americas. American Journal of Physical Anthropology 132:605-621.

Kemp BM, and Smith DG (2010) Ancient DNA Methodology: "Thoughts from Brian M. Kemp and David Glenn Smith on "Mitochondrial DNA of Protohistoric Remains of an Arikara Population from South Dakota". Human Biology 82:227-238.

Leney MD (2006) Sampling Skeletal Remains for Ancient DNA (aDNA): A Measure of Success. Historical Archaeology 40:31-49.

Malmstrom H, Stora J, Dalen L, Holmlund G, and Gotherstrom A (2005) Extensive human DNA contamination in extracts from ancient dog bones and teeth. Mol Biol Evol 22:2040-7.

Milos A, Selmanovic A, Smajlovic L, Huel RL, Katzmarzyk C, Rizvic A, and Parsons TJ (2007) Success rates of nuclear short tandem repeat typing from different skeletal elements. Croat Med J 48:486-93.

Misner LM, Halvorson AC, Dreier JL, Ubelaker DH, and Foran DR (2009) The correlation between skeletal weathering and DNA quality and quantity. J Forensic Sci 54:822-8.
Mundorff AZ, Bartelink EJ, and Mar-Cash E (2009) DNA preservation in skeletal elements from the World Trade Center disaster: recommendations for mass fatality management. J Forensic Sci 54:739-45.
NIJ (1999) What Every Law Enforcement Officer Should Know About DNA Evidence. National Institue of Justice.
NIJ (2005) Mass Fatality Incidents: A Guide for Human Forensic Identification. National Institue of Justice.
Pääbo S (1989) Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proc Natl Acad Sci U S A 86:1939-43.
Pääbo S, Gifford JA, and Wilson AC (1988) Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Research 16:9775-9788.
Parsons TJ, and Weedn VW (1997) Preservation and Recovery of DNA in Postmortem Specimens and Trace Samples. In WD Haglund and MH Sorg (eds.): Forensic Taphonomy: The Postmortem Fate of Human Remains. Boca Raton, Florida: CRC Press, pp. 109-138.
Prinz M, Carracedo A, Mayr WR, Morling N, Parson TJ, Sajantila A, Scheithauer R, Schmitter H, and Schneider PM (2007) DNA Commision of the International Society for Forensic Genetics (ISFG): Recommendations regarding the role of forensic genetics for disaster victim identification. Forensic Sci Int 1:3-12.
Rasband WS (1997-2012) ImageJ. Bethesda, MD: National Institutes of Health.
Salamon M, Tuross N, Arensburg B, and Weiner S (2005) Relatively well preserved DNA is present in the crystal aggregates of fossil bones. Proc Natl Acad Sci U S A 102:13783-13788.
Schwarz C, Debruyne R, Kuch M, McNally E, Schwarcz H, Aubrey AD, Bada J, and Poinar H (2009) New insights from old bones: DNA preservation and degradation in permafrost preserved mammoth remains. Nucleic Acids Res 37:3215-29.
Stoneking M, Hedgecock D, Higuchi RG, Vigilant L, and Erlich HA (1991) Population variation of human mtDNA control region sequences detected by enzymatic amplification and sequence-specific oligonucleotide probes. Am J Hum Genet 48:370-82.
Winters M, Barta JL, Monroe C, and Kemp BM (2011) To clone or not to clone: Method analysis for retrieving consensus sequences in ancient DNA samples. PLoS One 6:e21247.
Yang DY, and Watt K (2005) Contamination controls when preparing archaeological remains for ancient DNA analysis. Journal of Archaeological Science 32:331-336.

Table 1. Summary results, with intra-study ranking, of the genetic analysis of various skeletal elements. In the case the data from the Edson and colleagues (2004), Milos and colleagues (2007), and Mundorff and colleagues (2009) studies, "No. Successful" category reflects the number of specimens reported as "successful", regardless of the inter-study variation in the definition of success. The "No. Successful" for the Leney (2006) study was derived by rounding to the closest whole number of the sample size multiplied by reported percent successful. Studies by Edson and colleagues (2004) and Leney (2006) are based on mtDNA sequencing. As both of these studies originated from the Armed Forces DNA Identification Lab (AFDIL) it is possible that these data overlap. The study by Milos and colleagues (2007) is based on nuclear STR typing (Promega PowerPlex 16 System) and screening the amelogenin locus. The study by Mundorff and colleagues (2009) is based on nuclear DNA typing [Combined DNA Index System (CODIS) and two loci from Promega PowerPlex 16 System] and mtDNA sequencing.

For ease of viewing this table, it has been included as an excel file called "Appendix B" with this NIJ final report.

Table 2. Description of the 57 bone sections extracted, and copy number and inhibition information from the resulting extracts. Note that P=proximal, M=middle, and D=distal.

For ease of viewing this table, it has been included as an excel file called "Appendix C" with this NIJ final report.

Figure 1. Density Index (DI) measured on four bone portions. This image depicts both the sample with lowest (DI=0.39) and highest (DI=1.83) indices. Two addition samples are depicted to fill in the range.

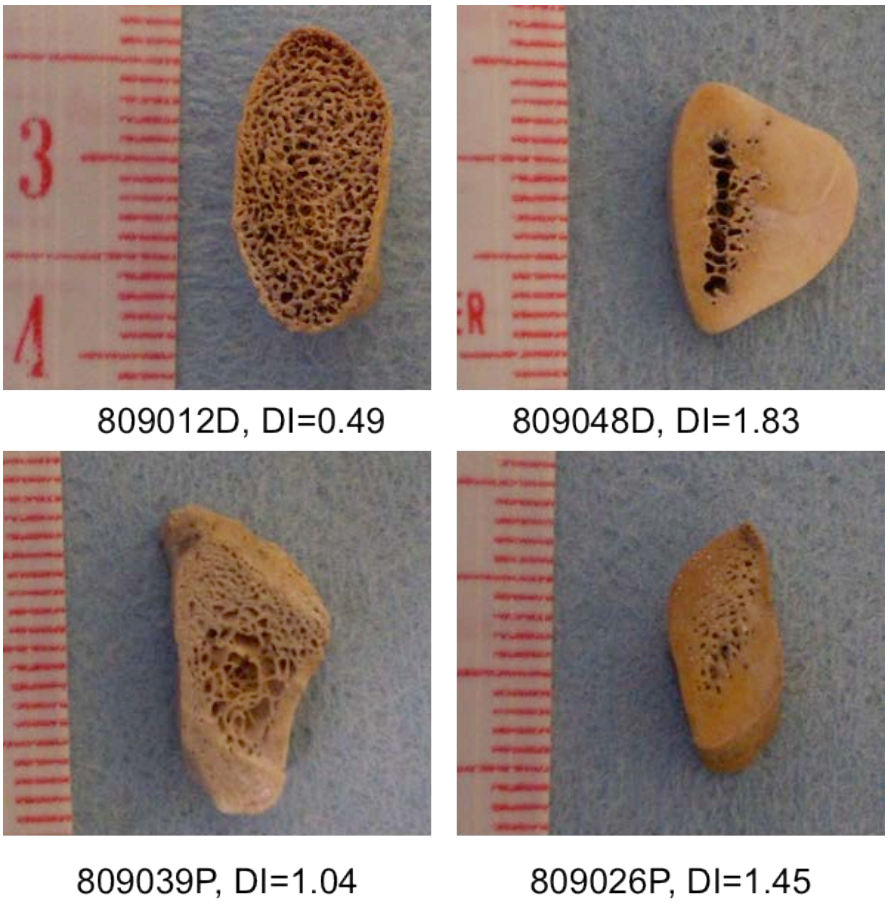
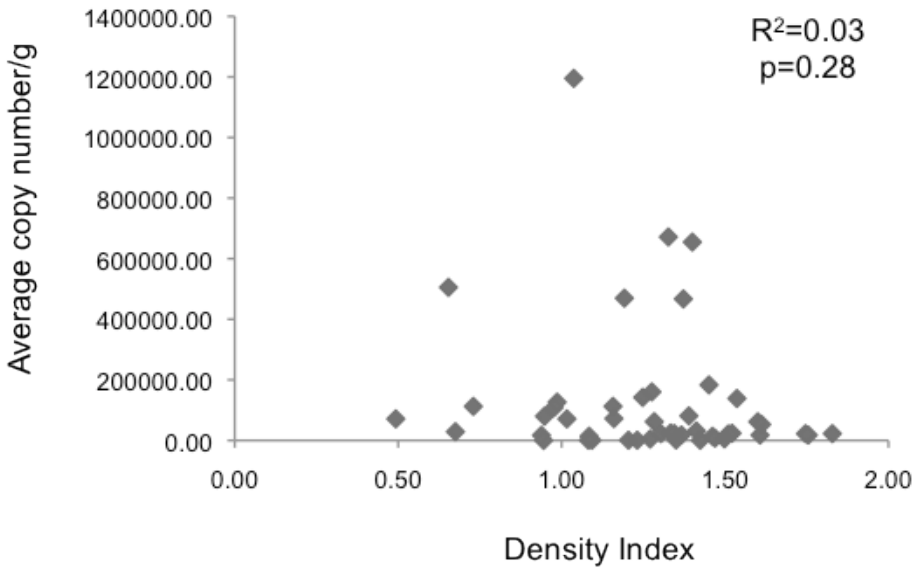


Figure 2. Results from the regression of density index against the average copy number/g.



One of the Key Characteristics of Ancient and Forensic DNA, Low Copy Number, May be a Product of its Extraction

Jodi Lynn Barta[a,b,c], Cara Monroe[a,b,d], Justin E. Teisberg[b], Misa Winters[a], Kelli Flanigan[e], and Brian M. Kemp[a,b,*]

[a] Department of Anthropology, Washington State University, Pullman, WA 99164-4236
[b] School of Biological Sciences, Washington State University, Pullman, WA 99164-4910
[c] Department of Biological and Health Sciences, Madonna University, Livonia, MI 48150
[d] Department of Anthropology, University of California-Santa Barbara, Santa Barbara, CA 93106-3210
[e] School of Molecular Biosciences, Washington State University, Pullman, WA 99164-7520

*Corresponding Author:

Brian M. Kemp
Department of Anthropology
Washington State University
Pullman, WA 99164
Office: 509-335-7403
Fax: 509-335-3999
bmkemp@wsu.edu

KEY WORDS: DNA extraction; Ancient DNA; Degraded DNA; Standards; DNA Loss

ABSTRACT

DNA from ancient and forensic sources is generally believed to be of low copy number, despite the previous lack of direct measurement of DNA loss accumulated through the extraction process. We developed synthesized "standards" to measure the efficiency of some common DNA extraction methods for degraded skeletal samples, and used quantitative PCR to estimate a known quantity of DNA subjected to a given extraction method (i.e. "copies in") versus quantity of DNA retained (i.e. "copies out"). All methods performed poorly in retaining short segments of DNA, giving low copy number results even when pre-extraction copy numbers far exceeded those expected of ancient samples. These findings challenge low copy number expectations, suggesting that ancient and forensic specimens may contain far more preserved genetic material than previously recognized. Furthermore, they emphasize the importance of optimizing and/or developing specialized extraction methods for retrieving degraded DNA.

INTRODUCTION

The observation that genetic material extracted from ancient specimens is typically both in a chemically degraded state and in low copy number was immediate (Pääbo, 1989; Pääbo et al., 1988) and has found little challenge in over 20 years of ancient DNA (aDNA) research. Advancements towards a greater understanding of the properties and "behavior" of degraded DNA (Gilbert et al., 2006; Schwarz et al., 2009) continue to underscore the difficulties inherent in the study of aDNA. Specifically, contaminating DNA from "modern" sources (e.g. Kemp and Smith, 2005), co-extracted impurities that inhibit PCR (Kemp et al., 2006) or influence extraction efficiency (Poinar et al., 1998), extraction conditions (Rohland and Hofreiter, 2007b), and/or simply the inability to free DNA from a sample can seriously jeopardize extraction success and authentication of results in ancient DNA studies. Similarly, these challenges are also met when working with low copy number (LCN[3]) and degraded DNA samples recovered from forensic contexts (Alaeddini et al., 2010; Alonso et al., 2004; Bär et al., 2000; Capelli et al., 2003; Edson et al., 2004; Jobling and Gill, 2004; von Wurmb-Schwark et al., 2008). In the case of forensic DNA analysis, both false positives (originating from contamination) and false negatives (arising, for example, from allelic drop-out) can compromise the strength of profiles recovered from such samples.

In the case of aDNA studies, the ability to generate authentic heterochronous sequence data is essentially what allows the field to continue to reveal novel insights about the evolutionary history of humans and other organisms, and long-term environmental change on earth (e.g. Green et al., 2010; Kemp et al., 2007; Krause et al., 2010; Lindqvist et al., 2010; Willerslev et al., 2007). Moreover, a phylochronological approach, studying genetic patterns across both time and space, permits more accurate assessment of specific changes in population histories than do studies of variation of contemporary gene pools alone (Hadly et al., 2004; Ramakrishnan and Hadly, 2009).

[3] In contrast to samples routinely studied by aDNA researchers, LCN DNA samples encountered in forensic investigations may represent those that begin from an initial deposition of only a minute amount of biological material, i.e. "trace DNA" or "touch DNA" (Lowe et al., 2002; van Oorschot et al., 2010), rather than those degraded to a LCN state. However, trace samples can certainly also be found in a chemically degraded state with regards to strand length (e.g. Hudlow et al., 2010).

Ancient DNA analysis provides a critical dimension because studies of contemporary variation do not detect lineage extinction (e.g. Malhi et al., 2007), which can be an integral part of a population's history.

Forensic DNA researchers immediately recognized the utility of analyzing genetic information from skeletal remains (Hagelberg et al., 1991; Jeffreys et al., 1992; Stoneking et al., 1991). Retrieving full or partial STR profiles from the nuclear genome, or even retrieving portions of mitochondrial DNA can prove invaluable in analysis of older cases (Nelson and Melton, 2007), war dead (Holland et al., 1993; Leney, 2006; Primorac et al., 1996), compromised samples (Foran, 2006), skeletal elements (Deng et al., 2005), missing persons (Bender et al., 2000), unknown victims (Erlich and Calloway, 2007), terrorist attacks (Mundroff et al., 2009), and other mass fatality incidents (Milos et al., 2007; Prinz et al., 2007; Zehner, 2007).

In either case, the benefits of studying ancient, LCN, and/or degraded DNA from skeletal samples are tempered by its challenging retrieval and authentication, principally because of postmortem damage sustained by the molecules that remain preserved in these specimens. Given the importance of information derived from skeletal sources, it is not surprising that a number of research teams, both from the aDNA and forensic DNA fields, have explored various DNA extraction methods to remove or avoid contamination and co-purify as few chemical impurities as possible while simultaneously retaining the maximum number of DNA molecules endogenous to the sample[4]. In general, this includes employing some combination of the following steps: 1) surface decontamination (in the case of bones and teeth Kemp and Smith, 2005), 2) demineralization and digestion of the material (i.e. with an extraction buffer and/or proteinase K), 3) phenol:chloroform extraction, 4) volume concentration (e.g. alcohol precipitation or size-exclusion filtration), and/or 5) a silica-based purification (e.g., Bolnick et al., 2012; Edson et al., 2004; Kemp et al., 2007; Misner et al., 2009; Pääbo, 1989; Pääbo et al., 1988; Rohland and Hofreiter, 2007a; Rohland et al., 2010; Yang et al., 1998).

With the goal of further maximizing success, researchers performed comparative studies of DNA yields using various extraction techniques on ancient and/or degraded specimens (e.g. Castella et al., 2006; Cattaneo et al., 1997; Davoren et al., 2007; Hoff-Olsen et al., 1999; Loreille et al., 2007; Rohland and Hofreiter, 2007b; Rohland et al., 2010; Yang et al., 1998). These studies often found one extraction method was superior to others tested under a specific set of conditions, such as the age and state of preservation of the biological material, and/or associated impurities in the samples. However, because these previous studies began with no knowledge of the *actual* DNA quantity in the samples prior to extraction, they ultimately compared the outcome of all methods *relative* to the best. While researchers may have identified the best extraction method within a candidate pool of methodologies, they could not determine how well these methods performed relative to complete recovery of DNA preserved in the material.

In order to expand our understanding of DNA loss, we assessed the performance variability of methods commonly used by aDNA and forensic DNA researchers.

[4] While numerous methods have been developed to improve downsteam analysis of ancient, LCN, and/or degraded DNA (e.g. Hudlow et al., 2008; Swango et al., 2007), the focus of our study is on the extraction of the DNA, the critical first step in any aDNA or forensic investigation.

Synthesized aDNA standards (defined and described below) were subjected to a variety of extraction methods prior to quantification by real-time PCR (qPCR). An estimate of how many copies of the standard (i.e., number of double stranded molecules) were available for "extraction" was compared to an estimate of the number of molecules retained throughout various steps of the extraction process individually, and the extraction processes as a whole. This approach provides as close to an *absolute* measure of extraction efficiency of the synthesized aDNA standards as possible and becomes an important tool in the assessment and validation of extraction methodologies. Our approach was not exhaustive to the number of extraction methods used across the field; those tested represent only a portion of them, which may be as numerous as there are laboratories working with degraded DNA (Anderung et al., 2008). We further note that the intention of the experiments conducted here was not to identify a superior method relative to others.

MATERIALS AND METHODS

DNA extraction of synthesized standards was conducted in a pre-PCR "modern" DNA laboratory separated from both the clean room (i.e. ancient DNA laboratory) and post-PCR facilities at Washington State University. PCR master reactions were assembled in the ancient DNA laboratory after which, DNA from extracted standards was added to the reactions in the pre-PCR "modern" DNA laboratory. This was to ensure that amplified DNA was not introduced into dedicated clean room facilities. Appropriate measures to minimize the introduction of contamination were employed by all researchers at each step of the protocol (Kemp and Smith, 2010).

Assessing Absolute Efficiency of DNA Extraction Methods Commonly Employed in the Study of Ancient, Degraded, and/or LCN Samples

Creating the Standards

Synthesized standards were created by amplifying modern northern fur seal (*Callorhinus ursinus)* blood plasma extracts for a 181 base pair (bp) portion of the mitochondrial cytochrome B gene, spanning nucleotide positions (nps) 14185-14365 [relative to a complete mtDNA genome, NC_008415 (Arnason et al., 2006)] with primers CytB-F CCAACATTCGAAAAGTTCATCC and CytB-R GCTGTGGTGGTGTCTGAGGT (Moss et al., 2006). We term these products "synthesized aDNA standards"; the short length of the molecules is intended to mimic the DNA yield from ancient and degraded samples (Pääbo, 1989). Typically, aDNA researchers have found that the majority of preserved DNA fragments are between 100 to 200 base pairs (bps) in length or shorter (see for example the quantitive PCR results of Poinar et al., 2006; Schwarz et al., 2009). Therefore, in this study, the synthesized aDNA standards were developed to represent a common upper size limit of aDNA. This size represents the mid-to-lower-range of those amplicons screened in commercial CODIS profiler kits [e.g. Promega PowerPlex 16® System (Krenke et al., 2002), ABI AmpFlSTR® Identifilier® (Collins et al., 2004)] and the mid-to-upper range of those amplicons targeted using a "miniSTR" approach to screen CODIS markers (Butler et al., 2003). Regardless, it is assumed that if this size fragment was not retained during a particular extraction process, neither would fragments of smaller size be retained. Moreover, using a non-human, non-domestic animal DNA standard for these experiments minimized the influence of possible contaminants. Following purification of the amplicons with a Qiagen PCR Purification Kit, standard

concentration was determined by spectrophotometry using a Nanodrop, copy numbers were calculated, and dilution series were performed to achieve a desired range of copy numbers in the aDNA standards.

Experimental Extractions

Numerous replicates of the various synthesized aDNA standards (SOM Table 1) were subjected to the following methods:

1) The extraction method described by Kemp and colleagues (2007) which combines a phenol:chloroform extraction, isopropanol precipitation, and silica-based purification.

    a) 100 µL of a synthesized aDNA standard was added to 2 mL 0.5 M EDTA (pH 8.0) in a 15 mL conical tube.
    b) 3 mg of proteinase K was added and the samples incubated at ~65°C for 3 hours.
    c) An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the sample, rocked for five minutes at room temperature and centrifuged at maximum speed for 10 minutes.
    d) The aqueous phase was transferred to a new 15 mL tube, to which an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The sample was then rocked for five minutes at room temperature and centrifuged at maximum speed for 10 minutes.
    e) The aqueous phase was transferred to a new 15 mL tube, to which an equal volume of chloroform:isoamyl alcohol :24:1) was added. The sample was then rocked for five minutes at room temperature and centrifuged at maximum speed for 10 minutes.
    f) The aqueous phase was transferred to a new 15 mL tube, to which one half volume of room temperature 5 M ammonium acetate was added. To this combined volume, one volume of room temperature absolute isopropanol was added. This solution was inverted 2-3 times and then stored at room temperature overnight.
    g) The tubes were centrifuged for 30 min at 3100 rpm to pellet the DNA. The liquid was poured off and the pellet left to air-dry for 15 min.
    h) The pellet was washed with 1 mL of 80% ethanol by vortexing (making sure to dislodge the pellet, if visible, from the side of the tube).
    i) The tubes were centrifuged for 30 min at 3100 rpm to pellet the DNA. The liquid was poured off and the pellet left to air-dry for 15 min.
    j) The pellet was rehydrated with 300 µL of DNA-free ddH_2O and purified with the Wizard PCR Preps DNA Purification System (Promega) using a vacuum manifold and following the manufacturer's instructions with the following modifications:

        i) The column was rinsed with 3 mL of 80% isopropanol.
        ii) DNA was removed from the column by incubating 50 µL of ~65°C DNA-free ddH_2O on the column for 3 min and then centrifuging the eluate at 10,000 x g for 30 sec. This step was repeated with an additional 50 µL of DNA-free ddH_2O collected in the same tube.

2) Phenol:chloroform extraction. This follows the three step phenol:chloroform protocol found within Kemp and colleagues (2007).

a) 300 µL phenol:chloroform:isoamyl alcohol (25:24:1) was added to 100 µL of a synthesized aDNA standard. This was mixed for 5 min on a rocker and centrifuged at maximum speed for 10 minutes.
b) The aqueous phase was transferred to a new tube and 300 µL phenol:chloroform:isoamyl alcohol (25:24:1) was added. This was mixed for 5 min on a rocker and centrifuged at maximum speed for 10 minutes.
c) The aqueous phase was transferred to a new tube and 300 µL chloroform:isoamyl alcohol (24:1) was added. This was mixed for 5 min on a rocker and centrifuged at maximum speed for 10 minutes.
d) The final aqueous phase was transferred to a new tube.

3) Isopropanol precipitation. This follows the isopropanol precipitation protocol found within Kemp and colleagues (2007).

a) 100 µL of a synthesized aDNA standard was added to 2 mL of DNA-free ddH_2O.
b) 1 mL of 5 M ammonium acetate and 3 mL of room temperature absolute isopropanol was added.
c) This solution was inverted 2-3 times and then stored at room temperature overnight.
d) The tubes were centrifuged for 30 min at 3100 rpm to pellet the DNA. The liquid was poured off and the pellet left to air-dry for 15 min.
e) The pellet was washed with 1 mL of 80% ethanol by vortexing (making sure to dislodge the pellet, if visible, from the side of the tube).
f) The tubes were centrifuged for 30 min at 3100 rpm to pellet the DNA. The liquid was poured off and the pellet left to air-dry for 15 min.
g) The pellet was rehydrated with 100 µL of DNA-free ddH_2O.

4) Concentration with microconcentrator.

a) 100 µL of a synthesized aDNA standard was added to 2 mL of DNA-free ddH_2O.
b) This mixture was concentrated with a small volume Millipore Microcon 30,000 MWCO (angled filter) microconcentrator to 100 uL following manufacturer's instructions.

5) Silica-based purification. These extractions utilized the Wizard PCR Preps DNA Purification System (Promega) using a vacuum manifold, with some modification described below.

a) Normal extraction.
   i) 100 µL of a synthesized aDNA standard was added to 1 mL of Wizard PCR Preps DNA Purification Resin. This mixture was vortexed numerous times over ~2 min period.
   ii) The mixture was pulled by vacuum across the Wizard minicolumn.
   iii) 3 mL of 80% isopropanol was pulled by vacuum across the column.
   iv) The column placed in a 1.5 mL tube and centrifuged at 10,000 x g for 2 min to remove any remaining isopropanol.
   v) The column was placed in a new 1.5 mL tube. DNA was finally removed from the column by allowing 50 µL of ~65°C DNA-free ddH_2O to sit on the column for 3 min and then centrifuged at 10,000 x g for 30 sec. This step was repeated with an additional 50 µL of DNA-free ddH_2O collected in the same tube.

b) Normal extraction with final elution in TE instead of water.
c) Extraction with variable amounts of Wizard PCR Preps DNA Purification Resin added: 250 µL, 500 µL, 750 µL, 1.25 mL. 1.5 mL, 1.75 mL, or 2 mL

d) Following the addition of variable amounts of Wizard PCR Preps DNA Purification Resin (250 µL, 500 µL, 750 µL, or 1.0 mL), 1 mL of 3M Sodium Acetate (pH 5.5) was added and the mixture was vortexed.

e) Following the addition of variable amounts of Wizard PCR Preps DNA Purification Resin (500 µL, 1.0 mL, or 2.0 mL), pH of mixture was adjusted to ~8.5 with 0.1 M NaOH.

f) Extraction with variable ratios of Wizard PCR Preps DNA Purification Resin to 6M guanindinium thiocyanate:
   i) 100 µL: 800 µL
   ii) 250 µL: 650 µL
   iii) 500 µL: 400 µL
   iv) 750 µL: 150 µL

6) Additional silica-based purifications, following manufacturer's instructions.

a) QIAquick PCR Purification Kit
b) MoBio -Ultra Clean DNA Puriifcation Kit
c) Geneclean-QBiogene

Quantification of Copy Number

Quantification PCRs were performed on all experimental extracts in an Applied Biosystems 7300 Real Time PCR System using a MAR-labeled probe (5'-CATTAACAGCTCGCTC-3', Allelogic) following Winters and colleagues (2011). Each 25 µL reaction contained 0.24 mM dNTPs, 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 0.24 µM probe, 0.5 µM ROX reference dye, 0.75 U of Platinum *Taq* polymerase (Invitrogen™), and 5.0 µL of template DNA. Cycling was performed with an initial 10 minute hold at 95°C followed by 50 cycles of 15 seconds at 95°C and 60 seconds at 55°C. A minimum of four PCR negative controls were included on each 96-well plate to monitor contamination in reagents and ROX-labeled passive reference dye was included to correct for variation in well-to-well background fluorescence. Amplification curves were analyzed with the manual baseline feature of the 7300 System SDS software (Applied Biosystems) with an empirically determined threshold of 0.05. Calibration curves were generated from a freshly prepared serial dilution series of standard DNA amplified from a modern northern fur seal plasma whole genomic DNA extraction (using the primers CytB-F and CytB-R, described above). Slopes of the calibration curves were used to calculate assay efficiencies (%PCR efficiency = $(10^{(-1/\text{slope})} - 1) \times 100$). A minimum efficiency > 87% with $R^2 > 0.996$ was set for data inclusion. Analyzed data were exported from the 7300 SDS software into a CSV file (comma delimited) for secondary analysis and formatting in Microsoft® Excel 2007.

For each run, synthesized aDNA standard extracts were quantified in duplicate to calculate a mean copy number count for the sample and its associated standard deviation. In the case that one of the duplicate reactions failed, the copy number of the

single observation was recorded for the run. The final copy number of the extract was calculated as an average of the run means, from which the standard deviation of copy number count was also calculated.

Calculating Efficiency

Estimations of the number of "copies in" were recorded as the average of 2-6 qPCR amplifications and standard deviations of these averages were also calculated.

Estimations of the number of "copies out" were recorded as the average of duplicate qPCR amplifications, except in the case that one of the two PCRs yielded results that were "undetermined" where the number was taken from a single qPCR measure. Standard deviations of the average numbers of "copies out" was also calculated in the cases where duplicate qPCR reactions yielded measures.

Subtraction of the number of copies out (measured as the average of duplicate PCR amplifications) from copies in (measured as the average of 2-6 PCR amplifications) divided by copies in provides the percent efficiency of each experimental method. The average of these efficiencies over all replicates provided an average efficiency. We also calculated the standard deviation of each average.

RESULTS AND DISCUSSION

The results of the experiments used to calculate extraction efficiency of the synthesized aDNA standards are found in SOM Table 1 and summarized in Tables 1 and 2. These experiments show that the typical methods for the extraction of ancient and degraded DNA from skeletal remains do not perform well in retaining short (i.e. 181 bp and, assumedly, molecules of shorter length) fragments. In the case of silica-based purifications (Table 2 and SOM Table 1), this does not agree with previous observations that DNA binding efficiency to silica is unrelated to molecule length (Melzak et al., 1996). Claims by manufacturers regarding retention rates for small sized DNA fragments require closer examination as they could be inflated by a failure to adequately measure the size and quantity of DNA pre- and post-extraction. To our knowledge these claims have not been based on results from methods similar to those conducted here. For the kits used in our study, the methods used by manufacturers to evaluate retention efficiencies and standard deviations are not described, nor are studies to support such claims cited in their respective manuals. Research focused on improving small sized molecule binding efficiency is essential to increase the retention of DNA recovered from ancient and degraded sources.

Results also highlight the fact that methods used for ancient and forensic DNA extraction from skeletal remains must strive to minimize the number of steps in the protocol, as DNA loss is compounded with each additional step. It is paradoxical in this case that some archaeological specimens demand extensive additional processing, for example "repeat silica extraction", to remove sufficient impurities before the endogenous DNA is accessible to the polymerase for amplification (Grier et al., 2013; Kemp et al., 2006).

Despite the recognized limitations of using a synthesized standard that mimics only the reality that forensic and aDNA tends to be degraded as it applies to fragment length, researchers that engage in developing, comparing or validating extraction methods are

encouraged to adopt a methodology similar to the one described here. At the very least, it provides a means to quantify "copies in" and "copies out" of the equivalent of "naked" DNA, giving, as close as possible, an *absolute* measure of retention to test the efficiency of the extraction methodology. To reiterate the point, in this study we have not exhaustively evaluated all available degraded DNA extraction methods. Far superior methods to those evaluated here may already exist, but using a standard approach as we have done here will be useful in determining how much better are these methods relative not only to one another, but also to complete recovery of DNA of some fragment length (which need not follow our lead with evaluation of a 181 bp fragment).

In addition, since all previous studies have estimated the quantity of DNA in specimens from the eluate at the end of an extraction process, the data presented here strongly suggest that previously observed copy numbers may be poor reflections of the overall amount of DNA actually preserved in ancient and degraded specimens. As a result, it is possible that these specimens could contain far more preserved genetic material than formerly recognized, and that low copy numbers result, in part, from the extraction procedures themselves.

Overall, this study demonstrates that low copy numbers, consistent with expectations for ancient and forensic DNA, can be created during the extraction of standards that far exceed most copy number expectations for an ancient sample (Table 1 and SOM Table 1). For example, extracting a synthesized standard containing 49194.25 copies/µL (SD 4430.94 copies/µL) can yield a mere 40.43 copies/µL (SD 19.69 copies/µL) (Table 1). While it is difficult to define what is considered an "acceptable" DNA copy number to be preserved in skeletal remains or where the cut off from ancient to "modern" DNA copy number begins[5], intuition plus two decades of reiteration has led to the general notion that there is very little DNA preserved in ancient specimens. Past research has taught many a valuable cautionary tale about paying attention to issues of contamination and the importance of authentication. However, lack of attention to some original assumptions may have resulted in a failure to fully understand DNA preservation in bone samples, and thus impeded progress. It is the ability to challenge these notions, and to question long held assumptions of what we think to be true, that will continue to move the field forward.

The synthesized standard used in these experiments mimics only fragment length degradation in forensic and aDNA. Other forms of post-mortem genetic damage, for example cross-linking (Poinar et al., 1998), and the complications of co-extracted chemical impurities routinely encountered in ancient and forensic DNA studies have yet to be addressed. While the realities of degradation make it highly unlikely that forensic or aDNA extraction copy numbers will ever reach "modern" levels, recognition that there is substantial preserved DNA in skeletal samples that is being lost during extraction is vital to improving methodologies for the extraction of DNA from finite resources such as ancient and forensic biological materials. Successfully accessing and retaining even a fraction of these DNA molecules will expand the potential of degraded materials research.

Even acknowledging the incredible research potential offered by the development of 2nd and 3rd generation sequencing does not change the fact that maximal retention of

[5] Forensic researchers have begun to address this issue with regards to low-template DNA profiles (Gill and Buckleton, 2010).

endogenous DNA molecules during extraction is an essential first step to capturing complete genomes and expanding data sets. Thus, the validity of manufacturer's size retention claims for devices commonly used in forensic and aDNA laboratories should be carefully examined while new attention should focus on maximizing DNA return by reducing the number of steps required during the extraction protocol, and by developing improved extraction methods whose efficiencies have been validated using a synthesized standard methodology.

ACKNOWLEDGMENTS

Thank you to Dr. Susan J. Crockford for turning our attention to the usefulness of ancient northern fur seals in these experiments. Special thanks to the Seattle Aquarium for providing modern northern fur seal samples to use for standards and positive controls. This project was supported by Award No. 2008-DN-BX-K008 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication/program/exhibition are those of the author(s) and do not necessarily reflect those of the Department of Justice. Support for Misa Winters and Kelli Flanigan was provided by the Interdisciplinary Training for Undergraduates in Biological and Mathematical Sciences (UBM) grant EF-0531870.

WORKS CITED

Alaeddini R, Walsh SJ, and Abbas A (2010) Forensic implications of genetic analyses from degraded DNA--a review. Forensic Sci Int Genet 4:148-57.

Alonso A, Martin P, Albarran C, Garcia P, Garcia O, de Simon LF, Garcia-Hirschfeld J, Sancho M, de la Rua C, and Fernandez-Piqueras J (2004) Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. Forensic Science International 139:141-149.

Anderung C, Persson P, Bouwman A, Elburg R, and Gotherstrom A (2008) Fishing for ancient DNA. Forensic Sci Int Genet 2:104-7.

Arnason U, Gullberg A, Janke A, Kullberg M, Lehman N, Petrov E, and Väinölä R (2006) Pinniped phylogeny and a new hypothesis for their origin and dispersal. Molecular phylogenetics and evolution 41:345-354.

Bär W, Brinkmann B, Budowle B, Carracedo A, Gill P, Holland M, Lincoln PJ, Mayr W, Morling N, Olaisen B, Schneider PM, Tully G, and Wilson M (2000) Guidelines for mitochondrial DNA typing. Vox Sanguinis 79:121-125.

Bender K, Schneider PM, and Rittner C (2000) Application of mtDNA sequence analysis in forensic casework for the identification of human remains. Forensic Sci Int 113:103-107.

Bolnick DA, Bonine HM, Mata-Miguez J, Kemp BM, Snow MH, and LeBlanc SA (2012) Nondestructive sampling of human skeletal remains yields ancient nuclear and mitochondrial DNA. Am J Phys Anthropol 147:293-300.

Butler JM, Shen Y, and McCord BR (2003) The Development of Reduced Size STR Amplicons as Tools for Analysis of Degraded DNA. Journal of Forenic Sciences 48:1054-1064.

Capelli C, Tschentscher F, and Pascali VL (2003) "Ancient" protocols for the crime scene? Similarities and differences between forensic genetics and ancient DNA analysis. Forensic Science International 131:59-64.

Castella V, Dimo-Simonin N, Brandt-Casadevall C, and Mangin P (2006) Forensic evaluation of the QIAshredder/QIAamp DNA extraction procedure. Forensic Sci Int 156:70-3.
Cattaneo C, Craig OE, James NT, and Sokol RJ (1997) Comparison of Three DNA Extraction Methods on Bone and Blood Stains up to 43 Years Old and Amplification of Three Different Gene Sequences. Journal of Forensic Sciences 42:1126-1135.
Collins PJ, Hennessy LK, Leibelt CS, Roby RK, Reeder DJ, and Foxall PA (2004) Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFlSTR Identifiler PCR Amplification Kit. J Forensic Sci 49:1265-77.
Davoren J, Vanek D, Konjhodzic R, Crews J, Huffine E, and Parsons TJ (2007) Highly effective DNA extraction method for nuclear short tandem repeat testing of skeletal remains from mass graves. Croat Med J 48:478-85.
Deng YJ, Li YZ, Yu XG, Li L, Wu DY, Zhou J, Man TY, Yang G, Yan JW, Cai DQ, Wang J, Yang HM, Li SB, and Yu J (2005) Preliminary DNA identification for the tsunami victims in Thailand. Genomics Proteomics Bioinformatics 3:143-57.
Edson SM, Ross JP, Coble MD, Parsons TJ, and Barritt SM (2004) Naming the Dead-Confronting the Realities of Rapid Identification of Degraded Skeletal Remains. Forensic Science Review 16:64-90.
Erlich H, and Calloway CD (2007) Using HLA and Mitochondrial DNA Polymorphisms to Identify Geographic/Ethnic Origins: The Mammoth Lakes Case. Forensic Magazine.
Foran DR (2006) Relative Degradation of Nuclear and Mitochondrial DNA: An Experimental Approach. J Forensic Sci 51:766-770.
Gilbert MT, Binladen J, Miller W, Wiuf C, Willerslev E, Poinar H, Carlson JE, Leebens-Mack JH, and Schuster SC (2006) Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis. Nucleic Acids Res 35:1-10.
Gill P, and Buckleton J (2010) A universal strategy to interpret DNA profiles that does not require a definition of low-copy-number. Forensic Sci Int Genet 4:221-7.
Green RE, Krause J, Briggs AW, Maricic T, Stenzel U, Kircher M, Patterson N, Li H, Zhai W, Fritz MH, Hansen NF, Durand EY, Malaspinas AS, Jensen JD, Marques-Bonet T, Alkan C, Prufer K, Meyer M, Burbano HA, Good JM, Schultz R, Aximu-Petri A, Butthof A, Hober B, Hoffner B, Siegemund M, Weihmann A, Nusbaum C, Lander ES, Russ C, Novod N, Affourtit J, Egholm M, Verna C, Rudan P, Brajkovic D, Kucan Z, Gusic I, Doronichev VB, Golovanova LV, Lalueza-Fox C, de la Rasilla M, Fortea J, Rosas A, Schmitz RW, Johnson PL, Eichler EE, Falush D, Birney E, Mullikin JC, Slatkin M, Nielsen R, Kelso J, Lachmann M, Reich D, and Paabo S (2010) A draft sequence of the Neandertal genome. Science 328:710-22.
Grier C, Flanigan K, Winters M, Jordan LG, Lukowski S, and Kemp BM (2013) Using Ancient DNA Identification and Osteometric Measures of Archaeological Pacific Salmon Vertebrae for Reconstructing Salmon Fisheries and Site Seasonality at Dionisio Point, British Columbia. Journal of Archaeological Science 40:544-555.
Hadly EA, Ramakrishnan U, Chan YL, van Tuinen M, O'Keefe K, Spaeth PA, and Conroy CJ (2004) Genetic response to climatic change: insights from ancient DNA and phylochronology. PLoS Biol 2:e290.
Hagelberg E, Gray IC, and Jeffreys AJ (1991) Identification of the skeletal remains of a murder victim by DNA analysis. Nature 352:427-9.

Hoff-Olsen P, Mevag B, Staalstrom E, Hovde B, Egeland T, and Olaisen B (1999) Extraction of DNA from decomposed human tissue. An evaluation of five extraction methods for short tandem repeat typing. Forensic Sci Int 105:171-83.
Holland MM, Fisher DL, Mitchell L, Rodriguez WC, Canick JJ, Merril CR, and Weedn VW (1993) Mitochondrial DNA sequence analysis of human skeletal remains: Identification of remains from the Vietnam War. Journal of Forensic Sciences 38:542-553.
Hudlow WR, Chong MD, Swango KL, Timken MD, and Buoncristiani MR (2008) A quadruplex real-time qPCR assay for the simultaneous assessment of total human DNA, human male DNA, DNA degradation and the presence of PCR inhibitors in forensic samples: a diagnostic tool for STR typing. Forensic Sci Int Genet 2:108-25.
Hudlow WR, Krieger R, Meusel M, Sehhat JC, Timken MD, and Buoncristiani MR (2010) The NucleoSpin(R) DNA Clean-up XS kit for the concentration and purification of genomic DNA extracts: an alternative to microdialysis filtration. Forensic Sci Int Genet 5:226-30.
Jeffreys AJ, Allen MJ, Hagelberg E, and Sonnberg A (1992) Identification of the skeletal remains of Josef Mengele by DNA analysis. Forensic Sci Int 56:65-76.
Jobling MA, and Gill P (2004) Encoded evidence: DNA in forensic analysis. Nat Rev Genet 5:739-51.
Kemp BM, Malhi RS, McDonough J, Bolnick DA, Eshleman JA, Rickards O, Martinez-Labarga C, Johnson JR, Lorenz JG, Dixon EJ, Fifield TE, Heaton TH, Worl R, and Smith DG (2007) Genetic analysis of early Holocene skeletal remains from Alaska and its implication for the settlement of the Americas. American Journal of Physical Anthropology 132:605-621.
Kemp BM, Monroe C, and Smith DG (2006) Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts. Journal of Archaeological Science 33:1680-1689.
Kemp BM, and Smith DG (2005) Use of Bleach to Eliminate Contaminating DNA from the Surfaces of Bones and Teeth. Forensic Science International 154:53-61.
Kemp BM, and Smith DG (2010) Ancient DNA Methodology: "Thoughts from Brian M. Kemp and David Glenn Smith on "Mitochondrial DNA of Protohistoric Remains of an Arikara Population from South Dakota". Human Biology 82:227-238.
Krause J, Fu Q, Good JM, Viola B, Shunkov MV, Derevianko AP, and Paabo S (2010) The complete mitochondrial DNA genome of an unknown hominin from southern Siberia. Nature 464:894-7.
Krenke BE, Tereba A, Anderson SJ, Buel E, Culhane S, Finis CJ, Tomsey CS, Zachetti JM, Masibay A, Rabbach DR, Amiott EA, and Sprecher CJ (2002) Validation of a 16-locus fluorescent multiplex system. J Forensic Sci 47:773-85.
Leney MD (2006) Sampling Skeletal Remains for Ancient DNA (aDNA): A Measure of Success. Historical Archaeology 40:31-39.
Lindqvist C, Schuster SC, Sun Y, Talbot SL, Qi J, Ratan A, Tomsho LP, Kasson L, Zeyl E, Aars J, Miller W, Ingolfsson O, Bachmann L, and Wiig O (2010) Complete mitochondrial genome of a Pleistocene jawbone unveils the origin of polar bear. Proc Natl Acad Sci U S A 107:5053-7.
Loreille OM, Diegoli TM, Irwin JA, Coble MD, and Parsons TJ (2007) High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int Genet 1:191-5.
Lowe A, Murray C, Whitaker J, Tully G, and Gill P (2002) The propensity of individuals to deposit DNA and secondary transfer of low level DNA from individuals to inert surfaces. Forensic Sci Int 129:25-34.

Malhi RS, Kemp BM, Eshleman JA, Cybulski J, Smith DG, Cousins S, and Harry H (2007) Haplogroup M Discovered in Prehistoric North America. Journal of Archaeological Science 34:642-648.
Melzak KA, Sherwood CS, Turner RFB, and Haynes CA (1996) Driving Forces for DNA Adsorption to Silica in Perchlorate Solutions. Journal of Colloid and Interface Science 181:635-644.
Milos A, Selmanovic A, Smajlovic L, Huel RL, Katzmarzyk C, Rizvic A, and Parsons TJ (2007) Success rates of nuclear short tandem repeat typing from different skeletal elements. Croat Med J 48:486-93.
Misner LM, Halvorson AC, Dreier JL, Ubelaker DH, and Foran DR (2009) The correlation between skeletal weathering and DNA quality and quantity. J Forensic Sci 54:822-8.
Moss M, Yang D, Newsome S, Speller C, McKechnie I, McMillan A, Losey R, and Koch P (2006) Historical ecology and biogeography of North Pacific pinnipeds: Isotopes and ancient DNA from three archaeological assemblages. The Journal of Island and Coastal Archaeology 1:165-190.
Mundroff AZ, Bartelink EJ, and Mar-Cash E (2009) DNA Preservation in Skeletal Elements from the World Trade Center Disaster: Recommendations for Mass Fatality Management. Journal of Forenic Sciences 54:739-745.
Nelson K, and Melton T (2007) Forensic mitochondrial DNA analysis of 116 casework skeletal samples. J Forensic Sci 52:557-61.
Pääbo S (1989) Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proc Natl Acad Sci U S A 86:1939-43.
Pääbo S, Gifford JA, and Wilson AC (1988) Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Research 16:9775-9788.
Poinar HN, Hofreiter M, Spaulding WG, Martin PS, Stankiewicz BA, Bland H, Evershed RP, Possnert G, and Paabo S (1998) Molecular coproscopy: Dung and diet of the extinct ground sloth Nothrotheriops shastensis. Science (Washington D C) 281:402-406.
Poinar HN, Schwarz C, Qi J, Shapiro B, Macphee RD, Buigues B, Tikhonov A, Huson DH, Tomsho LP, Auch A, Rampp M, Miller W, and Schuster SC (2006) Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA. Science 311:392-4.
Primorac D, Andelinovic S, Definisgojanovic M, Drmic I, Rezic B, Baden MM, Kennedy MA, Schanfield MS, Skakel SB, and Lee HC (1996) Identification of War Victims from Mass Graves in Croatia, Bosnia, and Herzegovina by the Use of Standard Forensic Methods and DNA Typing. Journal of Forensic Sciences 41:891-894.
Prinz M, Carracedo A, Mayr WR, Morling N, Parson TJ, Sajantila A, Scheithauer R, Schmitter H, and Schneider PM (2007) DNA Commision of the International Society for Forensic Genetics (ISFG): Recommendations regarding the role of forensic genetics for disaster victim identification. Forensic Sci Int 1:3-12.
Ramakrishnan U, and Hadly EA (2009) Using phylochronology to reveal cryptic population histories: review and synthesis of 29 ancient DNA studies. Mol Ecol 18:1310-30.
Rohland N, and Hofreiter M (2007a) Ancient DNA extraction from bones and teeth. Nat Protoc 2:1756-62.
Rohland N, and Hofreiter M (2007b) Comparison and optimization of ancient DNA extraction. Biotechniques 42:343-52.
Rohland N, Siedel H, and Hofreiter M (2010) A rapid column-based ancient DNA extraction method for increased sample throughput. Mol Ecol Resour 10:677-83.

Schwarz C, Debruyne R, Kuch M, McNally E, Schwarcz H, Aubrey AD, Bada J, and Poinar H (2009) New insights from old bones: DNA preservation and degradation in permafrost preserved mammoth remains. Nucleic Acids Res 37:3215-29.
Stoneking M, Hedgecock D, Higuchi RG, Vigilant L, and Erlich HA (1991) Population variation of human mtDNA control region sequences detected by enzymatic amplification and sequence-specific oligonucleotide probes. Am J Hum Genet 48:370-82.
Swango KL, Hudlow WR, Timken MD, and Buoncristiani MR (2007) Developmental validation of a multiplex qPCR assay for assessing the quantity and quality of nuclear DNA in forensic samples. Forensic Sci Int 170:35-45.
van Oorschot RA, Ballantyne KN, and Mitchell RJ (2010) Forensic trace DNA: a review. Investig Genet 1:14.
von Wurmb-Schwark N, Heinrich A, Freudenberg M, Gebuhr M, and Schwark T (2008) The impact of DNA contamination of bone samples in forensic case analysis and anthropological research. Legal Medicine 10:125-130.
Willerslev E, Cappellini E, Boomsma W, Nielsen R, Hebsgaard MB, Brand TB, Hofreiter M, Bunce M, Poinar HN, Dahl-Jensen D, Johnsen S, Steffensen JP, Bennike O, Schwenninger JL, Nathan R, Armitage S, de Hoog CJ, Alfimov V, Christl M, Beer J, Muscheler R, Barker J, Sharp M, Penkman KE, Haile J, Taberlet P, Gilbert MT, Casoli A, Campani E, and Collins MJ (2007) Ancient biomolecules from deep ice cores reveal a forested southern Greenland. Science 317:111-4.
Winters M, Barta JL, Monroe C, and Kemp BM (2011) To clone or not to clone: Method analysis for retrieving consensus sequences in ancient DNA samples. PLoS One 6:e21247.
Yang DY, Eng B, Waye JS, Dudar JC, and Saunders SR (1998) Technical note: Improved DNA extraction from ancient bones using silica-based spin columns. American Journal of Physical Anthropology 105:539-543.
Zehner R (2007) "Foreign" DNA in tissue adherent to compact bone from tsunami victims. Forensic Science International: Genetics 1:218-222.

Table 1. Average loss of ancient DNA standards using the extraction method of Kemp and colleagues (2007). This method combines a phenol:chloroform extraction, isopropanol precipitation, and a silica based purification. See SOM Table 1 for details and average loss of ancient DNA standards using other extraction methods.

Total Copy # In	SD	Copy #/uL In	SD	Total Copy # Out	SD	Copy #/uL Out	SD	Retention (%)	Loss (%)
64653	10781.04	646.53	107.81	35.60	26.97	3.56	2.70	0.06	99.94
177907	31882.00	1779.07	318.82	164.43	118.94	16.44	11.89	0.09	99.91
191603	5730.20	1916.03	57.30	5054.00	2997.26	505.40	299.73	2.64	97.36
4146910	99165.00	41469.10	991.65	1880.83	1342.72	188.08	134.27	0.05	99.95
4919425	443094.00	49194.25	4430.94	404.33	196.94	40.43	19.69	0.01	99.99

Table 2. Average loss of ancient DNA standards, ranging from 64653 (SD 10781) to 4919425 (SD 443094) "copies in", following various extraction methods.

Protocol		Average Loss (%)	SD
Kemp and colleagues (2007)		99.53	1.11
Phenol:Chloroform Extraction		59.16	5.65
Isopropanol Precipitation		99.35	0.15
Microconcentrator (Millipore)		79.50	24.13
Silica Purification (Promega Wizard)			
	following manufacturer's instructions	74.69	8.91
	with resin volume variation	45.06	21.34
	*with resin volume variation **and** sodium acetate pH adjustment*	61.66	18.61
	*with resin volume variation **and** NaOH pH adjustment*	82.53	10.81
	*with resin volume variation **and** additional 6M GuSCN*	48.62	26.19
	*with resin volume variation **and** additional 6M GuSCN **and** pH adjusted with HCl*	86.13	23.48
QIAquick PCR Purification Kit		71.25	30.56
MoBio		98.36	2.74
Geneclean		97.52	0.66

SOM Table 1. Result of the various experiments described in the main text.

For ease of viewing this table, it has been included as an excel file called "Appendix D" with this NIJ final report.

Evaluating the Efficacy of Various Thermo-Stable Polymerases Against Co-Extracted PCR Inhibitors in Ancient DNA Samples

Cara Monroe[a,b,c], Colin Grier[b], and Brian M. Kemp[a,b,*]

[a] Department of Anthropology, Washington State University**,** Pullman, WA 99164-4236
[b] School of Biological Sciences, Washington State University, Pullman, WA 99164-4910
[c] Department of Anthropology, University of California-Santa Barbara, Santa Barbara, CA 93106-3210

*Corresponding Author:

Brian M. Kemp
Department of Anthropology
Washington State University
Pullman, WA 99164
Office: 509-335-7403
Fax: 509-335-3999
bmkemp@wsu.edu

KEY WORDS: DNA extraction; Ancient DNA; Degraded DNA; Polymerase Chain Reaction Inhibitors; Polymerases; Species Identification

ABSTRACT

DNA from ancient and forensic specimens is often co-extracted with unknown amounts of unknown PCR inhibitors, which can lead to underestimated DNA concentrations, allelic drop-out, and/or false-negative results. It is not surprising, in this case, that numerous methods have been developed to remove PCR inhibitors or subdue their effects. One simple and cost effective approach could be the adoption of a polymerase that overcomes or is less affected by PCR inhibitors. In this study, nine different polymerases were evaluated for their efficacy against PCR inhibitors co-extracted with DNA from 63 ancient salmon vertebrae. These samples were excavated from two archeological sites located at the Dionisio Point locality on the northern end of Galiano Island in coastal southwestern British Columbia, Canada and date to 700-1000 and 1300-1500 years before present. Previously, DNA extracts from samples studied from this locality were determined to be largely inhibited to PCR amplificaiton. In the present study, Omni Klentaq LA (DNA Polymerase Technology, Inc.) outperformed the other 8 polymerases in two measures: 1) its success in genetic species identification of these vertebrae, and 2) its ability to amplify an ancient DNA positive control when spiked with a volume of inhibited extract from the vertebrae.

INTRODUCTION

Numerous substances can potentially inhibit PCR and are routinely encountered in both the study of ancient DNA (aDNA) (see review by Kemp et al., 2006) and forensic DNA (see review by Alaeddini, 2011). In these types of investigations, once contaminating DNA is sufficiently minimized or fully removed (e.g., Kemp and Smith, 2005), and because the degree of post-mortem damage (Lindahl, 1993; Molak and Ho, 2011) witnessed by a sample *cannot* be controlled, co-purified PCR inhibitors remain as the greatest threat to the successful study of ancient, degraded, and/or low copy number (LCN) DNA samples. The presence of PCR inhibitors can normally be confirmed visually as DNA extractions ranging from tinged yellow to dark brown in color (Figure 1). However, lack of coloration does not guarantee that a DNA extract is free of inhibitors (Alaeddini, 2011; Kemp et al., 2006).

The presence and concentration of PCR inhibitors have major consequences for downstream applications and can lead to underestimated DNA concentrations, allelic drop-out, and/or false-negative results (Funes-Huacca et al., 2011; King et al., 2009; Kontanis and Reed, 2006; Nolan et al., 2006; Opel et al., 2009). Compromised DNA material is particularly prone to inhibition, which can be introduced naturally through soils, plants (e.g. polysaccharides, humic, tannic, and fulvic acids), clothing (e.g. dyes, detergents) or food as well as during laboratory processing (phenol salts, ethanol, isopropanol, EDTA, and chaotrophic salts). Other and more insidious PCR inhibitors are endogenous to the biological samples themselves and include calcium ions and collagen from bone, blood components (billirubin, heme, immunoglobin, lactoferrin), salvia, semen, cervical fluid, bile, feces (polysaccharides, and humic acid), melanin, and myoglobin from muscle tissue among many others (Akane et al., 1994; Bélec et al., 1998; Demeke and Adams, 1992; Eckhart et al., 2000; Khan et al., 1991; Lantz et al., 1997; Monteiro et al., 1997; Scholz et al., 1998; Shutler et al., 1999; Tsai and Olson, 1992; Watson and Blackwell, 2000).

Given the importance of removing PCR inhibitors from DNA extracts a number of techniques have been developed to eliminate this problem. These methods can be

generally divided into two groups: (1) those that remove inhibitors during the DNA extraction and purification, and (2) those that diminish the effects of inhibitors by later manipulation of template DNA, PCR reagents, or incorporating PCR additives (reviewed by Alaeddini, 2011; Kemp et al., 2006).

While most of these techniques have been relatively effective with specific classes of inhibitors, it is difficult to predict which types of inhibitors or groups of inhibitors are present in any given sample (Bourke et al., 1999). Additionally, methods that remove inhibitors during extraction are associated with DNA loss, especially fragments <200 base pairs in length, which poses significant problem when working with degraded samples (Kemp et al., 2011) [see the study "One of the Key Characteristics of Ancient and Forensic DNA, Low Copy Number, May be a Product of its Extraction" above]. Moreover, some purification methods may achieve the desired end of removing environmental or endogenous PCR inhibitors while unintentionally incorporating new ones as a result of reagent carry-over (Bessetti, 2007; Boom et al., 1999).

Of the second category of techniques, diluting DNA and/or adding bovine serum albumin (BSA) to the PCR reaction are common remedies (Akane et al., 1994; Hummel and Herrmann, 1994; Martin, 2001; Shutler et al., 1999; Taylor and Swann, 1994). In the first instance PCR inhibitors are sufficiently diluted with water to no longer affect the PCR reaction, while at the same time the template DNA is not diluted sufficiently to preclude its amplification. As dilutions maintain a *constant* ratio of inhibitors to DNA, the effectiveness of this approach must be contingent on either: 1) the existence of an inhibitor threshold level, or 2) creating sufficient distance in the solution between inhibitory molecules (in this case referring to inhibitors not bound directly to the DNA) and template DNA, thus subduing their effects. Overcoming inhibitors through dilution may not be the best approach as it increases the need for experimental PCR reactions in order to determine appropriate dilutions. Moreover, it may be counterproductive to dilute template DNA already present in low copy number, as is found in degraded samples (Ye et al., 2004). Similarly, BSA blocks PCR inhibitors and, thus indirectly promotes polymerase activity. However, the use of BSA suffers from complications comparable to serial dilutions. Determining the appropriate concentrations is problematic, as various types of inhibitors require differing amounts of BSA in order to overcome PCR inhibition and in some cases BSA is ineffective or can even be detrimental to the success of an amplification (Taylor et al., 1997).

As a result it is preferable to have a generalized technique that decreases time, cost, and labor, and one that eliminates, circumvents, and/or inactivates as many inhibitors as possible while maintaining DNA yield. Adopting such a strategy will ultimately be a cost effective means for working with DNA in the face of PCR inhibitors.

A simple solution might be the adoption of the polymerase that performs well in the face of inhibitory molecules. For example, a number of studies have shown that polymerase derived from *Thermus aquaticus* (*Taq*) is highly susceptible to inhibitory substances whereas polymerases from *Pyrococcus furiosus* (*Pfu*), *Pyrococcus woesei* (*Pwu*), *Thermus flavus* (*Tfl*), and *Thermus thermophilus* (*Tth and rTth*) are less so (Al-Soud et al., 2000; Al-Soud and Radstrom, 1998; Al-Soud and Radstrom, 2000; Katcher and Schwartz, 1994; Wiedbrauk et al., 1995). For example, *Tth* polymerase was used to successfully amplify DNA in the presence of immunoglobin, myoglobin, bile, ocular fluid and phenol (Al-Soud et al., 2000; Al-Soud and Radstrom, 1998; Bélec et al., 1998; Katcher and Schwartz, 1994) and *Pwo* polymerase was able to overcome inhibition from

blood (Al-Soud and Radstrom, 1998). However, while some polymerases have higher fidelity over *Taq* polymerase, as well as higher tolerances for certain inhibitors, they can produce lower yields as a consequence of their 3'- 5' exonuclease (proofreading) activity (Pavlov et al., 2004).

Researchers often overcome this proclivity of *Taq* polymerase by adding increasing amounts to PCR reactions (Edwards et al., 2004; Fisher et al., 1993; Pääbo et al., 1988; Sutloviæ et al., 2005). While, increasing the amount of *Taq* polymerase may be an effective means to amplify DNA in the face of inhibition, this practice simultaneously makes a system that is already hypersensitive to the detection of contamination even more so (Kemp et al., 2006; Yang et al., 2003) and, as a result, can become cost and time ineffective.

The extent to which alternative forms of polymerase can tolerate inhibitory substances as well as amplify degraded samples is only beginning to be understood in aDNA and forensic studies. It is therefore compelling to determine which polymerase or manufactured blends of polymerases give the most optimal yield, fidelity, and resistance to a wide-range of inhibitors. In this project we evaluated the overall effectiveness of nine different thermo-stable polymerases and polymerase blends in their ability to amplify mitochondrial DNA (mtDNA) present in DNA extractions from salmon vertebrae from two archaeological sites (DgRv-003 and DgRv-006). These samples were chosen for two reasons. First, in a previous study of salmon vertebrae from the DgRv-003 site, DNA extracted from the samples was found to be particularly challenging to purify (Grier et al., 2013), requiring on average 4.62 (SD= 2.31) repeat silica extractions to sufficiently remove the inhibitory effects (following Kemp et al., 2006). In our experience, these samples are some of the most inhibited samples we have ever worked with, rivaling even ~6000-9000 year old human fecal samples (or "coprolites") from archaeological sites located in Southeastern Utah (Kemp et al., 2010). Secondly, working with non-human, non-domestic animal samples minimizes the influence that contamination might otherwise have had. In the end, we posed a simple question—that is, which of a number of commercially available polymerases or blends of polymerases performs best in the study of these highly inhibited samples. Our approach was not exhaustive to the number of polymerases available on the market; those tested represent only a portion of them. Performances were measured very simply as percent return of salmonid mtDNA sequences and percent of samples that amplified when spiked with an aDNA positive control.

MATERIALS AND METHODS

Archaeological Materials

Salmon vertebrae were recovered from excavations at two archaeological sites located within the Dionisio Point locality on the northern end of Galiano Island in coastal southwestern British Columbia, Canada (Grier et al., 2013). Twenty-two of the vertebral elements analyzed in this study derive from excavations within an ancient plankhouse (House 2) at the DgRv-003 (or "Dionisio Point") archaeological site, dated to approximately 1300-1500 years ago. The other forty-one samples analyzed in this study were recovered from the remains of a second plankhouse (House 1) at the DgRv-006 site, which dates to between 700-1000 years ago The two sites are in adjacent bays and separated by roughly 150 meters.

The salmon elements analyzed were sampled from much larger zooarchaeological assemblages. On the whole, bone was well preserved at both sites, at least visually. Despite the difference in age, the state of preservation appears comparable between sites. Because of their proximity, soil, moisture conditions and geology are similar, and bone elements were likely subject to similar anthropogenic and cultural processes at both sites.

DNA Methods

All preparation methods (i.e., extraction and PCR set-up) were conducted in the aDNA laboratory at Washington State University, one dedicated to the analysis of degraded and low copy number (LCN) DNA. Appropriate measures to minimize contamination and, importantly, to detect it if present, were employed (Kemp and Smith, 2010).

DNA Extraction

Except in one case, portions of each vertebra, weighing between 12-195 mg (Table 1), were carefully removed from the whole using a fresh razor blade on each sample. In the case of sample #410 from DgRv-003, which weighed 10 mg, DNA was extracted from the whole sample. Samples were extracted in batches of seven with an accompanying extraction negative control to which no sample was added. Samples were submerged in 6% w/v sodium hypochlorite (Clorox bleach) for four minutes to remove contaminating DNA from the surfaces of the samples (Kemp and Smith, 2005). The samples were then rinsed twice with DNA-free ddH_2O and DNA was extracted according to a modified protocol of Kemp et al. (2007) changed specifically in the silica extraction portion of the method as follows: 1) following the isopropanol precipitation, 750 µL of 2% celite in 6M guanidine HCl[6] and 250 µL of 6M guanidine HCl were added to samples and vortexed numerous times over a 2 minute period, 2) 3 mL of DNA-free ddH_2O was pulled across the syringe and Promega Wizard® Minicolumns to wash them before pulling the samples across the columns, 3) the DNA bound silica was rinsed with 3 mL of 80% isopropanol (versus 2 mL recommended by the manufacturer), 4) 50 µL of 65ºC DNA-free ddH_2O was added to the column and left for 3 minutes prior to centrifugation, and this step was repeated again resulting in 100 µL of extracted DNA.

Polymerases and Salmonid mtDNA PCRs

Nine different polymerases were tested in this study: 1) Amplitaq Gold® DNA Polymerase (Invitrogen), 2) Herculase II Fusion DNA Polymerase (Agilent), 3) Omni Klentaq LA (DNA Polymerase Technology, Inc.), 4) Phusion® High Fidelity DNA Polymerase (Finnzymes), 5) Platinum® Taq DNA Polymerase (Invitrogen), 6) *Pwo* DNA Polymerase (Roche), 7) *rTth* (Applied Biosystems) 8) *Tfl* DNA Polymerase (Promega), and 9) $Vent_R$® DNA Polymerase (New England Biolabs). From this point onward, these polymerases will simply be referred to as: 1) Amplitaq Gold, 2) Herc II, 3) Klentaq, 4)

[6] This solution is intended to mimic the Wizard® PCR Preps DNA Purification Resin, as best could be ascertained from the *Material Safety Data Sheet* (*MSDS*). To make this solution, add DNA-free ddH_2O to 1.25 mg of Celite® Analytical Filter Aid II (CAFA II, Sigma) up to 25mL, vortex and let incubate at room temperature overnight. Pour off the water carefully as to not pour off the celite and add DNA-free ddH_2O to 5mL and 6M guanidine HCl (Teknova) to 50mL.

Phusion, 5) Platinum Taq, 6) *Pwo*, 7) *rTth*, 8) *Tfl*, and 9) Vent. All PCR amplifications were conducted in 15 μL reaction volumes that included 1.5 μL of template DNA. Details of the PCR reactions conducted in this study are summarized in Table 2. Attempts were made to standardize PCR components across kits and make the reactions match as closely to those described by Kemp et al. (2007) which used Platinum Taq and has been met with success across a number of additional studies of ancient bones, teeth, and feces (e.g. Bolnick et al., 2012; Malhi et al., 2007; Speller et al., 2010; Wilson et al., 2011; Winters et al., 2011). Complete standardization was not always possible in the cases that cofactors were already premixed in the PCR buffers at various concentrations (i.e., for Herc II and Klentaq, Table 2). Moreover, we did not control for differences between the components found in the PCR buffers supplied with the polymerases, which are highly variable (see respective manufacturer's manuals).

A 189 base pair (bp) portion of the 12S mitochondrial gene, used for species identification of salmonids, was PCR amplified in 15 μL reactions according to Table 2, using the primers (called "OST12S-F" and "OST12S-R") described by Jordan et al. (2010). PCR denaturing and extension conditions are summarized in Table 2 and varied according the manufacturer's recommendations for the respective polymerases. A rainbow trout (*Oncorhynchus mykiss*) positive control, added in the post-PCR lab just prior to running the PCRs, accompanied each batch of reactions to preclude PCR failure. The extraction negative controls and PCR negatives were tested in parallel with all rounds of amplification.

Four microliters of PCR products were separated on 6% polyacrylamide gels. Gels were stained with ethidium bromide and viewed under UV light to confirm successful amplification. Amplicons were prepared for sequencing by addition of 10 U of *ExoI* and 2 U of SAP. Reactions were incubated at 37º C for 20 min, followed by 80ºC for 20 min. Sequences were generated in both directions at Elim Biopharm (Hayward, CA). Aligned against a rainbow trout mtDNA sequence (NCBI Accession DQ288271) in Sequencher (version 4.8), sequences generated in this study were used for species determination according to Jordan et al. (2010).

Evaluating Inhibitory Effects

Extractions and modified extractions (as described below) were tested for inhibitory effects against the polymerases listed above, following the rationale provided by Kemp and colleagues (2006) and as put into practice by Grier and colleagues (2013) and Moss and colleagues (In Preparation) using DNA extracted from ~170-415 year old goose remains (Wilson et al., 2011) as the "positive aDNA controls." However, in contrast to those previous studies, here batches of seven to thirteen goose bones were extracted, as described above, and pooled into what we call "Goose Collective" ancient positive controls. The choice to pool these individual extractions was intentionally done to even out variance across samples in both endogenous goose mtDNA copy number and possible inhibitors co-extracted with the goose DNA. A different Goose Collective was used in each of the three rounds of experiments described below, so that the inhibitory effect of the salmonid extractions is directly comparable within each round.

Fifteen microliter PCRs, which included 1.5 μL of "Goose Collective" DNA, were set up according to Table 2 to amplify a 159 bp portion of goose mitochondrial cytochrome B gene using the primers "BSP-I" and "GooseR" described by Wilson and colleagues

(2011). To these reactions, 1.5 μL of the ancient salmon template DNA was added (totaling 16.5 μL reactions). The extraction negative controls were also tested for inhibitors. PCRs were run according to Table 2 in parallel with a goose PCR that contained no salmon DNA extract (this reaction was used as a positive control, which allowed us to preclude PCR failure from contributing to our results (Kemp and Smith, 2010). PCR negatives accompanied each round of amplification. If the goose DNA failed to amplify when spiked with ancient salmon DNA extract, we considered the polymerase to be inhibited, regardless of whether the ancient salmon mtDNA amplified as described above. If the goose DNA amplified when spiked with ancient salmon DNA extract, but exhibited a noticeably dimmer band, we considered this to be "slightly" inhibited, and recorded it as such.

Experiments

During the course of the experiments described below, we recognized that it is crucial that the polymerases tested here are able to amplify the ancient "Goose Collective" positive control alone. Otherwise, they could not be tested for inhibitors as designed in this study (described above under "*Evaluating Inhibitory Effects"*), regardless of their ability to amplify ancient salmonid mtDNA. In this case, both Amplitaq Gold and *Tfl* failed to be able to amplify the "Goose Collective" positive control and were, therefore, removed from the study.

In the first round of experiments, we tested Klentaq, Platinum Taq, and *Pwo* against one another in their ability amplify ancient salmonid mtDNA from 21 extracts, and 1:10 and 1:50 dilutions of those extractions. In the second round of experiments, we tested Klentaq (the best performing polymerase in the first round of experiments), Herc II, and Vent against one another in their ability amplify ancient salmonid mtDNA from an additional 21 extracts, and 1:10 and 1:50 dilutions of those extractions. In the last round of experiments, we tested Klentaq (the best performing polymerase in the first two rounds of experiments), Phusion and *rTth* against one another in their ability amplify ancient salmonid mtDNA from a third set of 21 extracts, and 1:10 and 1:50 dilutions of those extractions.

In each set of experiments the color of the full concentration and diluted DNA extractions were visually inspected against a plain white paper background and recorded (Figure 1 and Table 1).

Data Analysis

The success of the various polymerases in all of the experiments was based on two measures: 1) the number of positive returns of salmon species identifications, and 2) the number of extracts that were not affected by inhibition (in this case, "slightly" inhibited results were treated as successes). The number of successes was totaled in each experimental round, across the full concentration extractions and dilutions of those extracts. Two-tailed Fisher's exact tests were used to statistically evaluate differences in performance between the polymerases.

RESULTS AND DISCUSSION

Because both Amplitaq Gold and *Tfl* failed to be able to amplify the "Goose Collective" positive control they were removed from further consideration in this study. We were

particularly surprised that Amplitaq Gold failed in this experiments, as it is a widely used polymerase in aDNA studies (e.g. Haak et al., 2010; Rohland and Hofreiter, 2007; Shapiro, 2008; Yang et al., 2003).

In the first round of experiments, of the 21 full concentration extracts neither Klentaq, Platinum *Taq*, nor *Pwo* were capable of amplifying salmonid mtDNA from the full concentration extractions (Table 1). Klentaq and Platinum *Taq* were inhibited by all 21 extracts, whereas *Pwo* was not inhibited by 2/21 (9.5%) of the extracts. We note also that the extraction negative control 1/13/12-A that accompanied the second batch of extractions was inhibited to all three polymerases. This observation likely stems from the unintentional carry-over of some chemical through the extraction process (e.g. alcohol and/or phenol). This was the only extraction negative control that behaved in this manner.

In the case of the 1:10 dilutions of the extractions, Klentaq yielded two positive results, one chum salmon (*O. keta*) and one sockeye salmon (*O. nerka*). From the extract of sample #336, it also amplified a 183 bp (including primers) section of human DNA corresponding to chromosome 2 [according to a NCBI Blast search (Altschul et al., 1997), Accession number AC237676.1). This is not too surprising, as the primers "OST12S-F" and "OST12S-R") designed by Jordan et al. (2010) have been shown to be capable of amplifying human and mouse (*Mus musculus*), but not brown bear (*Ursus arctos*) or dog (*Canis lupus familiaris*) DNA (Grier et al., 2013). Platinum *Taq* also yielded two positive results, first confirming the observation that sample 420 from DgRv-003 is a chum salmon and secondly identifying sample 401 from DgRv-003 as likely pink salmon (*O. gorbuscha*). The sequence exhibited a thymine (T) at nucleotide position (np) 660 and a T at np 670, relative to the rainbow trout mtDNA sequence. Since the 670T has not yet been observed among contemporary salmonids (Jordan et al., 2010) or ancient salmonids (Grier et al., 2013; Moss et al., In Preparation), it likely represents post-mortem genetic damage [the most common form of miscoding lesions leads to artificial C>T "transitions" (Gilbert et al., 2007)]. Nevertheless, the sequence exhibits no other known shared-derived mutations that would cause the samples to be identified as any Pacific salmonid species besides pink. *Pwo* was incapable of amplifying any salmonid DNA in the 1:10 dilutions. Klentaq was not inhibited by 12/21 of the extracts (~57.1%) and showed to be "slightly" inhibited by the extraction negative control 1/13/12-A. Platinum *Taq* was not inhibited by 3/21 of the extracts (~14.3%), and also not by extraction negative control 1/13/12-A. *Pwo* was not inhibited by 13/21 of the extracts (~61.9%), and also not by extraction negative control 1/13/12-A.

In the case of the 1:50 dilutions of the extractions, Klentaq yielded the same two positive results but did not amplify any human contamination from sample #336, whereas Platinum *Taq* yielded only one positive result and amplified human DNA in one PCR negative control [matching a human BAC clone, according to an NCBI Blast search (Altschul et al., 1997)], Accession number AC092107.5]. *Pwo* was again incapable of amplifying any salmonid DNA from these dilutions even though they were able to amplify the ancient goose positive control. Klentaq was the least inhibited (20/21, ~95.2% of extracts not inhibited), followed by *Pwo* (18/21, ~85.7% of extracts not inhibited) and Platinum *Taq* (12/21, ~57.1% of extracts not inhibited).

Across these extracts and their dilutions, there was no difference (at the 0.05 level of significance level) in positive returns between Klentaq (4/63 successes) Platinum Taq (3/63 successes) (p=1.00). Interestingly, both polymerases were used to successfully

identify the species of a sample that the other did not produce: 1) sample #405 was identified sockeye by Klentaq, but not Platinum *Taq*, and 2) sample #401 as pink by Platinum *Taq*, but not by Klentaq. However, Klentaq was significantly less inhibited (32/63) in this round of experiments than Platinum *Taq* (17/63) (p=0.0102). While *Pwo* yielded zero successes in amplifying salmonid mtDNA in this set of experiments, it was less inhibited (31/63) than Platinum *Taq* (p=0.0167), but not different than Klentaq (p=1.00).

In the second round of experiments, of the 21 full concentration extracts, neither Klentaq (the best performing polymerase in the first round of experiments), Herc II, nor Vent were capable of amplifying salmonid mtDNA and all were deemed to be inhibited.

In the case of the 1:10 dilution of these extracts: 1) Klentaq returned positive results from 2/21 extracts (~9.5%) and was not inhibited by 1/21 extracts (~4.8%), 2) Herc II returned positive results from 1/21 samples (~4.8%), and was not inhibited by 1/21 extracts (~4.8%), and 3) Vent was not capable of amplifying salmonid and all extracts were deemed to be inhibited.

In the case of the 1:50 dilution of these extracts Klentaq returned positive results from 12/21 extracts (~57.1%) and was not inhibited by 15/21 extracts (~71.4%). One of the positive results, sample #56 from site DgRv-006 was identified as sockeye salmon, but exhibited a mix of cytosine (C) and thymine (T) at np 650 relative to the rainbow trout mtDNA reference sequence. This is likely the result of sequencing a mix of damaged and undamaged template molecules. It also produced a double-banded amplicon from sample 72. The lower band appeared to be approximately the correct size, with the upper band being ~20 bps larger. The sequence returned from these amplicons was unreadable. Herc II returned positive results from 6/21 extracts (~28.6%) and was not inhibited by 1/21 extracts (~4.8%). Vent was again not capable of amplifying salmonid mtDNA and 6/21 (~28.6%) of the 1:50 diluted extracts were inhibited.

Across these extracts and their dilutions, Klentaq yielded the highest return on salmon species identifications (14/63 successes), however it was not statically greater than the return from Herc II (7/63) (p=0.1504). Yet, in no case was Herc II used to make a species identification that was not made by Klentaq. Conversely Klentaq was used to uniquely identify the species of 6 salmon vertebrae in this round of experiments. Klentaq was less inhibited (16/63) than both Herc II (2/63) (p=0.0006) and Vent (6/63) (p=0.0330)

In the third round of experiments, of the 21 full concentration extracts, neither Klentaq (the best performing polymerase in the first and second rounds of experiments), Phusion, nor *rTth* were capable of amplifying salmonid mtDNA and all were deemed to be inhibited. In addition, *rTth* was inhibited by one of the extraction negative controls (2/10/12-F).

In the case of the 1:10 dilution of these extracts, Klentaq was used to identify 11/21 samples (~52.4%) as salmonids. In addition sample #57 produced a sequence that is 7 mutational steps away from a number of different fishes according to an NCBI Blast search (Altschul et al., 1997). However, this sample is tentatively typed as Pacific greenling (*Hexagrammos sp.*) and this observation is treated as a positive result (totaling 12/21, or ~57.1%). Klentaq was not inhibited by 13/21 samples (~61.9%). Phusion returned positive results from 3/21 samples (~14.3%) and was inhibited by all of the 1:10

diluted extracts. *rTth* was not capable of amplifying salmonid mtDNA and all were deemed to be inhibited.

In the case of the 1:50 dilution of these extracts, Klentaq was used to identify 18/21 samples (~85.7%) as being salmonids. One of these samples (#49) was identified as sockeye salmon, but exhibits additional transitions [to adenine (A)] at nucleotide positions (nps) 690 and 703 relative to the rainbow trout mtDNA sequence. As Klentaq was used to make this same species identification from the 1:10 dilution of this extract but that sequence did not exhibit the 690A and 703A mutations, it is concluded that these "mutations" are due to damaged template molecules detected in the 1:50 dilution. Since these artifactual mutations appear without competing secondary peaks, it suggests that that PCR was initiated from very few molecules. Given that this extract is five times more dilute than the 1:10 diluted extract, this observation makes sense. Sample #57 produced again the "Pacific greenling-like" sequence, identical to that observed in the 1:10 diluted extract, bringing the total success for Klentaq to 19/21 (90.5%). Klentaq was not inhibited by 20/21 samples (~95.2%). Phusion returned positive results from 10/21 samples (~47.6%, including samples #57 with an identical "Pacific greenling-like" sequence) and was not inhibited by 4/21 of the 1:50 diluted extracts (~19%). In addition it produced a 359 bp amplicon (including primers) from sample #1, which was unidentifiable according to a NCBI Blast search (Altschul et al., 1997). *rTth* returned positive results from 3/21 samples (~14.3%) and was not inhibited by 2/21 of the extracts (9.5%).

Across these extracts and their dilutions, Klentaq (31/63 successes) outperformed Phusion (13/63) (p=0.0014) and *rTth* (3/63) (p=0.0001) in its use for species identification among these 21 vertebrae. Klentaq was also far less susceptible to inhibition (33/63 successes) compared with Phusion (4/63) (p=0.0001) and *rTth* (2/63) (p=0.0001).

Considering the whole of the results reported here, it is important to note that our experimental approach to testing for inhibition produced results that require further assessment to account for the following. First, in the first round of experiments, 1:10 dilute extracts that amplified successfully for salmonid mtDNA (i.e. sample #420 with Klentaq and samples #401 and #420 with Platinum Taq) demonstrated to be inhibited against amplifying the Goose Collective ancient positive control. Across the extractions that yielded 75 positive species identifications, 19 of them (~25.3%) were also determined to be inhibited. This was observed in 8/13 (~61.5%) successes using Phusion, 6/7 (~85.7%) using Herc II, 2/3 (~66.6%) using Platinum Taq, 2/49 (~4.1%) using Klentaq, and 1/2 (50%) using *rTth* DNA Polymerase, XL. While the outcomes of our experimental design suggest further tests are warranted, it is still instructive to the extent that it was designed for evaluating the efficacies of the polymerase tested here.

With one exception (sample #337 extract with *Pwo*), all of the full concentration extracts were determined to be inhibitory to all of the polymerases tested in this study. That 8 of 63 of these extracts were visibly colorless (Table 1) further demonstrates that a pigment free extractions does not guarantee that they are free of inhibitors. Conversely, all 55 full concentration extracts exhibiting coloration, ranging from being very lightly tinged to dark brown in color (Table 1), were inhibited, demonstrating that any degree of pigmentation in an extract is a good predictor of co-extracted PCR inhibitors.

CONCLUSIONS

Overall, Omni Klentaq LA outperformed the other 8 polymerases in two respects: 1) its successful use in genetic species identification of these vertebrae, and 2) its ability to amplify an ancient DNA positive control when spiked with a volume of inhibited extract from the vertebrae. Therefore, Klentaq appears particularly well suited to situations were DNA is co-extracted with PCR inhibitors from bone samples. However, we temper this recommendation with the following observations: 1) that Klentaq did not statistically outperform either Platinum Taq or Herc II in positive species identifications, and 2) that in one instance Platinum Taq was used to make a species identification that was not achieved with Klentaq. Yet, at the time of writing this manuscript, Omni Klentaq LA is the most cost effective choice at $0.43/U (when 625U are purchased) compared to Platinum *Taq* ($0.86/U, when 500U are purchased) and Herc II ($0.46, when 500U are purchased).

ACKNOWLEDGMENTS

This project was supported by Award No. 2008-DN-BX-K008 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication/program/exhibition are those of the author(s) and do not necessarily reflect those of the Department of Justice. Thanks to Erin Reams for assistance in the laboratory.

WORKS CITED

Akane A, Matsubara K, Nakamura H, Takahashi S, and Kimura K (1994) Identification of the Heme Compound Copurified with Deoxyribonucleic Acid (DNA) from Bloodstains, a Major Inhibitor of Polymerase Chain Reaction (PCR) Amplification. Journal of Forensic Sciences 39:362-372.

Al-Soud WA, Jonsson LJ, and Radstrom P (2000) Identification and Characterization of Immunoglobulin G in Blood as a Major Inhibitor of Diagnostic PCR. Journal of Clinical Microbiology 38:345-350.

Al-Soud WA, and Radstrom P (1998) Capacity of Nine Thermostable DNA Polymerases To Mediate DNA Amplification in the Presence of PCR-Inhibiting Samples. Applied and Environmental Microbiology 64:3748-3753.

Al-Soud WA, and Radstrom P (2000) Effects of Amplification Facilitators on Diagnostic PCR in the Presence of Blood, Feces, and Meat. Journal of Clinical Microbiology 38:4463-4470.

Alaeddini R (2011) Forensic implications of PCR inhibition--A review. Forensic Sci Int Genet 6:297-305.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.

Bélec L, Authier J, Eliezer-Vanerot M-C, Piédouillet C, Mohamed AS, and Gherardi RK (1998) Myoglobin as a polymerase chain reaction (PCR) inhibitor: A limitation for PCR from skeletal muscle tissue avoided by the use of thermus thermophilus polymerase. Muscle & Nerve 21:1064-1067.

Bessetti J (2007) Profiles in DNA (Promega) 10:9-10.

Bolnick DA, Bonine HM, Mata-Miguez J, Kemp BM, Snow MH, and LeBlanc SA (2012) Nondestructive sampling of human skeletal remains yields ancient nuclear and mitochondrial DNA. Am J Phys Anthropol 147:293-300.

Boom R, Sol C, Beld M, Weel J, Goudsmit J, and Wertheim-van Dillen P (1999) Improved silica-guanidiniumthiocyanate DNA isolation procedure based on selective binding of bovine alpha-casein to silica particles. J Clin Microbiol 37:615-9.

Bourke MT, Scherczinger CA, Ladd C, and Lee HC (1999) NaOH treatment to neutralize inhibitors of Taq polymerase. Journal of Forensic Sciences 44:1046-1050.

Demeke T, and Adams RP (1992) The effects of plant polysaccharides and buffer additives on PCR. Biotechniques 12:332-334.

Eckhart L, Bach J, Ban J, and Tschachler E (2000) Melanin binds reversibly to thermostable DNA polymerase and inhibits its activity. Biochem Biophys Res Commun 271:726-730.

Edwards CJ, MacHugh DE, Dobney KM, Martin L, Russell N, Horwitz LK, McIntosh SK, MacDonald KC, Helmer D, Tresset A, Vigne J-D, and Bradley DG (2004) Ancient DNA analysis of 101 cattle remains: limits and prospects. Journal of Archaeological Science 31:695-710.

Fisher DL, Holland MM, Mitchell L, Sledzik PS, Wilcox AW, Wadhams M, and Weedn VW (1993) Extraction, evaluation, and amplification of DNA from decalcified and undecalcified United States Civil War Bone. Journal of Forensic Sciences 38:60-68.

Funes-Huacca ME, Opel K, Thompson R, and McCord BR (2011) A comparison of the effects of PCR inhibition in quantitative PCR and forensic STR analysis. Electrophoresis 32:1084-9.

Gilbert MT, Binladen J, Miller W, Wiuf C, Willerslev E, Poinar H, Carlson JE, Leebens-Mack JH, and Schuster SC (2007) Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis. Nucleic Acids Res 35:1-10.

Grier C, Flanigan K, Winters M, Jordan LG, Lukowski S, and Kemp BM (2013) Using Ancient DNA Identification and Osteometric Measures of Archaeological Pacific Salmon Vertebrae for Reconstructing Salmon Fisheries and Site Seasonality at Dionisio Point, British Columbia. Journal of Archaeological Science 40:544-555.

Haak W, Balanovsky O, Sanchez JJ, Koshel S, Zaporozhchenko V, Adler CJ, Der Sarkissian CS, Brandt G, Schwarz C, Nicklisch N, Dresely V, Fritsch B, Balanovska E, Villems R, Meller H, Alt KW, and Cooper A (2010) Ancient DNA from European early neolithic farmers reveals their near eastern affinities. PLoS Biol 8:e1000536.

Hummel S, and Herrmann B (1994) General Aspects of Sample Preparation. In S Hummel and B Herrmann (eds.): Ancient DNA: Recovery and Analysis of Genetic Material from Paleontological, Archaeological, Museum, and Forensic Specimens. New York: Springer-Verlag, pp. 59-68.

Jordan LG, Steele CA, and Thorgaard GH (2010) Universal mtDNA primers for species identification of degraded bony fish samples. Mol Ecol Resour 10:225-8.

Katcher HL, and Schwartz I (1994) A distinctive property of Tth DNA polymerase: enzymatic amplification in the presence of phenol. Biotechniques 16:84-92.

Kemp BM, Barta JL, Monroe C, Teisberg J, Runnells S, and Flanigan K (2011) One of the Key Characteristics of Ancient DNA, Low Copy Number, May be a Product of its Extraction: 80th Annual Meeting of the American Association of Physical Anthropologists. Minneapolis, MN.

Kemp BM, Malhi RS, McDonough J, Bolnick DA, Eshleman JA, Rickards O, Martinez-Labarga C, Johnson JR, Lorenz JG, Dixon EJ, Fifield TE, Heaton TH, Worl R, and Smith DG (2007) Genetic analysis of early Holocene skeletal remains from Alaska and its implication for the settlement of the Americas. American Journal of Physical Anthropology 132:605-621.

Kemp BM, Monroe C, Geib PR, Matson RG, and Lipe WD (2010) Genetic Analysis of Coprolites from Southeastern Utah. 75th Annual Meeting of the Society for American Archaeology.

Kemp BM, Monroe C, and Smith DG (2006) Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts. Journal of Archaeological Science 33:1680-1689.

Kemp BM, and Smith DG (2005) Use of Bleach to Eliminate Contaminating DNA from the Surfaces of Bones and Teeth. Forensic Science International 154:53-61.

Kemp BM, and Smith DG (2010) Ancient DNA Methodology: "Thoughts from Brian M. Kemp and David Glenn Smith on "Mitochondrial DNA of Protohistoric Remains of an Arikara Population from South Dakota". Human Biology 82:227-238.

Khan G, Kangro HO, Coates PJ, and Heath RB (1991) Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. J Clin Pathol 44:360-5.

King C, Debruyne R, Kuch M, Schwarz C, and Poinar H (2009) A quantitative approach to detect and overcome PCR inhibition in ancient DNA extracts. Biotechniques 47:941-9.

Kontanis EJ, and Reed FA (2006) TECHNICAL NOTE Evaluation of Real-Time PCR Amplification Efficiencies to Detect PCR Inhibitors. Journal of Forensic Sciences 51:795.

Lantz P-G, Matsson M, and Radstrom P (1997) Removal of PCR inhbitors from human faecal samples through the use of an aqueous tow-phase system for sample preparation prior to PCR. Journal of Microbiological Methods 28:159-167.
Lindahl T (1993) Instability and decay of the primary structure of DNA. Nature 362:709-715.
Malhi RS, Kemp BM, Eshleman JA, Cybulski J, Smith DG, Cousins S, and Harry H (2007) Haplogroup M Discovered in Prehistoric North America. Journal of Archaeological Science 34:642-648.
Martin LR (2001) STR-Typing Of Nuclear DNA From Human Fecal Matter Using The Qiagen Qiaamp® Stool Mini Kit. Twelfth International Symposium on Human Identification.
Molak M, and Ho SY (2011) Evaluating the impact of post-mortem damage in ancient DNA: a theoretical approach. J Mol Evol 73:244-55.
Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R, Cabrita J, and Megraud F (1997) Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. J Clin Microbiol 35:995-8.
Moss ML, Judd KG, and Kemp BM (In Preparation) Can salmonids (Oncorhynchus spp.) be identified to species using vertebral morphometrics? A test using ancient DNA. Journal of Archaeological Science.
Nolan T, Hands RE, Ogunkolade W, and Bustin SA (2006) SPUD: a quantitative PCR assay for the detection of inhibitors in nucleic acid preparations. Anal Biochem 351:308-10.
Opel KL, Chung D, and McCord BR (2009) A study of PCR inhibition mechanisms using real time PCR. J Forensic Sci 55:25-33.
Pääbo S, Gifford JA, and Wilson AC (1988) Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Research 16:9775-9788.
Pavlov AR, Pavlova NV, Kozyavkin SA, and Slesarev AI (2004) Recent developments in the optimization of thermostable DNA polymerases for efficient applications. Trends Biotechnol 22:253-260.
Rohland N, and Hofreiter M (2007) Comparison and optimization of ancient DNA extraction. Biotechniques 42:343-52.
Scholz M, Giddings I, and Pusch CM (1998) A polymerase chain reaction inhibitor of ancient hard and soft tissue DNA extracts is determined as human collagen type I. Analytical Biochemistry 259:283-286.
Shapiro B (2008) Engineered polymerases amplify the potential of ancient DNA. Trends Biotechnol 26:285-7.
Shutler GG, Gagnon P, Verret G, Kalyn H, Korkosh S, Johnston E, and Halverson J (1999) Removal of a PCR inhibitor and resolution of DNA STR types in mixed human-canine stains from a five year old case. Journal of Forensic Sciences 44:623-626.
Speller CF, Kemp BM, Wyatt SD, Monroe C, Lipe WD, Arndt UM, and Yang DY (2010) Ancient Mitochondrial DNA Analysis Reveals Complexity of Indigenous North American Turkey Domestication. Proceedings of the National Academy of Science USA 107:2807-2812.
Sutloviæ D, Gojanoviæ MD, Anðelinoviæ Š, Gugiæ D, and Primorac D (2005) Taq Polymerase Reverses Inhibition of Quantitative Real Time Polymerase Chain Reaction by Humic Acid. Croat Med J 46:556-562.
Taylor GM, Rutland P, and Molleson T (1997) A sensitive polymerase chain reaction method for the detection of Plasmodium species DNA in ancient human remains. Ancient Biomolecules 1:193-203.

Taylor JW, and Swann EC (1994) DNA from Herbarium Specimens. In S Hummel and B Herrmann (eds.): Ancient DNA: Recovery and Analysis of Genetic Material from Paleontological, Archaeological, Museum, and Forensic Specimens. New York: Springer-Verlag, pp. 166-181.

Tsai Y-L, and Olson BH (1992) Rapid Method for Separation of Bacterial DNA from Humic Substances in Sediments for Polymerase Chain Reaction. Applied & Environmental Microbiology 58:2292-2295.

Watson RJ, and Blackwell B (2000) Purification and characterization of a common soil component which inhibits the polymerase chain reaction. Can J Microbiol 46:633-42.

Wiedbrauk DL, Werner JC, and Drevon AM (1995) Inhibition of PCR by aqueous and vitreous fluids. Journal of Clinical Microbiology 33:2643-2646.

Wilson BW, Crockford SJ, Johnson JW, Malhi RS, and Kemp BM (2011) Genetic and archaeological evidence for a former breeding population of Aleutian cackling goose, Branta hutchinsii leucopareia, on Adak Island, central Aleutians, Alaska. Canadian Journal of Zooology:732-743.

Winters M, Barta JL, Monroe C, and Kemp BM (2011) To clone or not to clone: Method analysis for retrieving consensus sequences in ancient DNA samples. PLoS One 6:e21247.

Yang DY, Eng B, and Saunders SR (2003) Hypersensitive PCR, ancient human mtDNA, and contamination. Human Biology 75:355-364.

Ye J, Ji AQ, Parra EJ, Zheng XF, Jiang CT, Zhao XC, Hu L, and Tu Z (2004) A simple and efficient method for extracting DNA from old and burned bone. Journal of Forensic Sciences 49:754-759.

Figure 1. Photograph depicting, in the left seven tubes in the top row, DNA extractions from salmonid vertebrae (samples 1, 21, 49, 75, 94, 160, and 167) recovered from the Late period plankhouse at the DgRv-006 site. These samples date to approximately 1000 to 700 cal BP. Dilutions of these samples are found in the left seven tubes of the middle row (1:10) and the bottow row (1:50). The eighth tube in each row represents the extraction negative control and dilutions of that control (1:10 and 1:50). This batch of samples illustrates the range of inhibitors visual observed in this study (and the range we have typically observed in other studies). In the top row, from left to right the samples were described as follows: (1) light brown, (2) brown, (3) light brown, (4) dark, dark brown, (5) brown, (6) light brown, (7) light brown, and (8) clear. In the middle row, from left to right the 1:10 dilutions were described as follows: (1) clear, (2) light tinge, (3) clear, (4) brown, (5) light tinge, (6) clear, (7) clean, and (8) clear. In the bottom row, from left to right the 1:50 dilutions were described as follows: (1) clear, (2) clear, (3) clear, (4) tinged, (5) clear, (6) clear, (7) clean, and (8) clear.

Table 1. Samples studied and results. Note: X=amplification, O=no amplification

For ease of viewing this table, it has been included as an excel file called "Appendix E" with this NIJ final report.

Table 2. Specification of PCR components and conditions of the salmonid mtDNA reactions conducted in this study. All reactions were 15μL in volume, employed primers "OST12S-F" and "OST12S-R" (Jordan et al., 2010), and were run for 60 cycles. The PCR reactions used to test for inhibition were set-up as described for salmonid mtDNA, employed primers "BSP1" and "GooseR" (Wilson et al., 2011), but were spiked with 1.5μL of "Goose Collective" ancient positive control (resulting in total volume of 16.5μL). Sixty cycles of touch-down PCR, during which the initial annealing temperature of 60°C was reduced by 0.1°C each round, were conducted according to Wilson et al. (2011).

	PCR Components								
	Amplitaq Gold	**Herculase II**	**Omni Klentaq**	**Phusion**	**Platinum *Taq***	***Pwo***	***rTth***	***Tfl***	**Vent**
dNTPs	0.32 mM	0.32 mM	0.32 mM	0.32 mM	0.32 mM	0.32 mM	0.32 mM	0.32 mM	0.32 mM
Buffer	1X PCR Gold Buffer	1X Herculase II Reaction Buffer (including final concentration of 2mM $MgCl_2$)	1X Omni Klentaq Reaction Buffer (including final concentration of 3.5mM $MgCl_2$)	1X Phusion® HF Buffer	1X PCR Buffer	1X PCR Buffer	1X XL Buffer II	1X Reaction Buffer	1X ThermoPol Reaction Buffer
Cofactor	1.5 mM $MgCl_2$	In buffer	In buffer	1.5 mM $MgCl_2$	1.5 mM $MgCl_2$	1.5 mM $MgSO_4$	1.5 mM $Mg(OAc)_2$	1.5 mM $MgSO_4$	1.5 mM $MgSO_4$
Primers	0.24 μM each	0.24 μM each	0.24 μM each	0.24 μM each	0.24 μM each	0.24 μM each	0.24 μM each	0.24 μM each	0.24 μM each
Polymerase	0.3 U	0.3 U	0.3 U	0.3 U	0.3 U	0.3 U	0.3 U	0.3 U	0.3 U
Template DNA	1.5 μL	1.5 μL	1.5 μL	1.5 μL	1.5 μL	1.5 μL	1.5 μL	1.5 μL	1.5 μL
	PCR Reactions								
	Amplitaq Gold	**Herculase II**	**Omni Klentaq**	**Phusion**	**Platinum *Taq***	***Pwo***	***rTth***	***Tfl***	**Vent**
Initial Denaturing	95°C/5 min	95°C/2 min	94°C/3 min	98°C/30 sec	94°C/3 min	94°C/3 min	94°C/1 min	94°C/30 sec	95°C/2 min
Denaturing	95°C/15 sec	95°C/15 sec	95°C/15 sec	98°C/15 sec	94°C/15 sec	94°C/15 sec	94°C/15 sec	95°C/15 sec	95°C/15 sec
Annealing	55°C/15 sec	55°C/15 sec	55°C/15 sec	55°C/15 sec	55°C/15 sec	55°C/15 sec	55°C/15 sec	55°C/15 sec	55°C/15 sec
Extension	72°C/15 sec	72°C/15 sec	68°C/15 sec	72°C/15 sec	72°C/15 sec	72°C/15 sec	72°C/15 sec	74°C/15 sec	72°C/15 sec
Final Extension	72°C/3 min	72°C/3 min	68°C/3 min	72°C/3 min	72°C/3 min	72°C/3 min	72°C/3 min	74°C/3 min	72°C/3 min

Evaluating the Behavior and Degree of Post-Mortem Damage in Ancient DNA Template Molecules

Brian M. Kemp[a,b,*] and Cara Monroe[a,b,c]

[a] Department of Anthropology, Washington State University**,** Pullman, WA 99164-4236
[b] School of Biological Sciences, Washington State University, Pullman, WA 99164-4910
[c] Department of Anthropology, University of California-Santa Barbara, Santa Barbara, CA 93106-3210

*Corresponding Author:

Brian M. Kemp
Department of Anthropology
Washington State University
Pullman, WA 99164
Office: 509-335-7403
Fax: 509-335-3999
bmkemp@wsu.edu

KEY WORDS: Post-mortem Genetic Damage; Ancient DNA; Degraded DNA; Behavior of Degraded DNA; qPCR

ABSTRACT

DNA extracted from 127 ancient human bone, teeth, and coprolite (i.e., feces) samples was amplified for eleven fragments varying in lengths from 62-1144 base pairs (bps) to evaluate the "behavior" and degree of post-mortem damage of ancient DNA (aDNA) template molecules. Of the 211 sequences not compromised by contamination, 34 (16.1%) exhibited miscoding lesions presumably due to post-mortem damage. Observation of this form of damage in direct sequences is positively related to amplicon length, which indirectly suggests that it is negatively related to starting template copy number. We have termed this phenomenon as "frequency dependent damage detection" (or FD^3), which is a characteristic of aDNA that may become useful in the authentication of future aDNA results. However, where quantitative PCR (qPCR) results were generated in this study, they demonstrated no statistical difference in mean starting copy number for sequences that exhibited damage versus those that did not display damage (p=0.25 for 166 base pair (bp) amplicon, p=0.486 for 215 bp amplicon). Moreover, none of our PCRs were initiated from >1000 molecules, demonstrating that this cut-off, while regularly cited, is arbitrary for generating authentic aDNA results. Lastly, in this study we observed intact aDNA molecules in excess of 500 bps in length and found the contamination in this study to be both more degraded with regards to strand length and lower in copy number than some aDNA samples. Combined with observations from previous studies, the degree of post-mortem damage appears to sample specific, making it difficult to generalize for all aDNA specimens and/or rely on a single set of recommendations as a means for absolute authentication.

INTRODUCTION

The key to the success of any ancient DNA (aDNA) study relies on the authentication of results. While true of any line of scientific inquiry, conclusions drawn from the study of aDNA are only as strong as data generated to support them. Since DNA extracted from ancient remains is typically recovered in low copy number and is short with regards to strand length (see review by Gilbert, 2006; Pääbo, 1989; Pääbo et al., 1988), the success of aDNA studies can be highly compromised by contamination originating from "modern" sources (Kemp and Smith, 2005; Malmstrom et al., 2005; Yang and Watt, 2005) and PCR inhibitors (Kemp et al., 2006). Moreover, ancient template molecules often exhibit further chemical modifications (e.g., miscoding lesions) that have occurred post-mortem (Gilbert et al., 2003a; Gilbert et al., 2003b; Hofreiter et al., 2001a; Pääbo, 1989). If gone unrecognized, this form of damage can result in artificial "mutations" that can skew estimates of genetic diversity and mutation rates, and lead to problems in inferring demographic histories (Axelsson et al., 2008; Ho et al., 2007a; Ho et al., 2007b; Rambaut et al., 2009). The degree to which these problems will influence any one study varies, with the study of ancient human DNA arguably being of the highest risk (Gilbert et al., 2005).

Investigations of forensic and/or low copy number (LCN) DNA samples suffer similar problems of contamination, PCR inhibition, and post-mortem damage (Alaeddini, 2011; Alaeddini et al., 2010; Alonso et al., 2004; Bär et al., 2000; Capelli et al., 2003; Edson et al., 2004; Jobling and Gill, 2004; von Wurmb-Schwark et al., 2008). While over a decade ago, Fattorini and colleagues (2000) showed that reliable allele-specific oligonucleotide (ASO) probing could not be conducted on some forensic samples due to "artifacts" in the degraded DNA, today forensic DNA researchers and those faced with interpreting sequences from such aged and degraded specimens need a full

appreciation for the extent of post mortem genetic damage. This topic has been approached by the forensic DNA community (Fattorini et al., 1999; Fattorini et al., 2000; Previdere et al., 2002), however recent discussion has been dominated by ancient DNA researchers (however, see the excellent review by Alaeddini et al., 2010). While artifactual mutations encountered in aDNA studies might distort our reconstruction of the evolution of a species, their potential impact in forensic investigations is markedly more alarming. Thus, knowledge of the degree of damage exhibited by aged and/or degraded remains will guide interpretation, for example, of mitochondrial DNA (mtDNA) mismatches to comparative samples, as has been needed to account for the high mutation rate of the genome and prevalence of heteroplasmy (Buckleton et al., 2005; Tully et al., 2001).

In response to the recognized problems associated with the study of aDNA, a number of researchers have made recommendations to others in the field on how to properly conduct research and, in turn, authentic results (e.g., Cooper and Poinar, 2000; Gilbert et al., 2005; Kemp and Smith, 2010; Pääbo et al., 2004; Willerslev and Cooper, 2005; Winters et al., 2011). While the views of these researchers vary, one point that appears to be widely agreed upon across the field is that aDNA should exhibit *appropriate molecular behavior*. On account of its degraded state, aDNA "behaves" differently than does "modern" DNA in experiments. This observation stems from the seminal work of Pääbo and colleagues (1988) and Pääbo (1989) in which a strong negative relationship between amplicon size and PCR efficiency was noted. Quantitative behavioral differences between ancient and modern DNA continues to be used as a means of authenticating results (e.g., Malmstrom et al., 2009; Malmstrom et al., 2007; Ottoni et al., 2009; Schwarz et al., 2009), while some researchers will simply use the failure to produce large amplicons as evidence that their data are derived from authentic aDNA molecules (e.g., Caramelli et al., 2003; Caramelli et al., 2008; Hekkala et al., 2011; Lawrence et al., 2010; Wang et al., 2007). Regardless of the approach taken, a confounding factor is that PCR inhibitors have been shown to preferentially affect amplification of larger fragments [(Pusch and Bachmann, 2004), but see Deagle (2006)]. Another important aspect of the appropriate molecular behavior of aDNA is that, due to differences in copy number, nuclear DNA results should only be obtainable from ancient remains when the analysis of mtDNA is possible (Cooper and Poinar, 2000).

Given the agreement that aDNA should behave in a certain manner, one might expect that there should be some agreement over just how intact are these template molecules. In other words—*How degraded are aDNA molecule supposed to be?* The answer to this question is important as it could serve as another point of authentication for aDNA results, however a review of the literature reveals quite a range of answers. Many of the estimates for the average aDNA strand length are found in review papers. For example, O'Rourke and colleagues (2000: pg 219) stated "recovery and amplification of aDNA, when possible, is usually limited to fragments <300–500 bp in length, and only for samples in the range of tens of thousands, or fewer, years old." These authors cite no evidence for this claim. Citing Pääbo (1989) and Hofreiter and colleagues (2001b), Pääbo and colleagues (2004: pg 647) stated "The most obvious type of damage to DNA extracted from subfossil and fossil remains is its degradation to small average size, generally between 100 to 500 bp." It is worth noting that in their review, Hofreiter and colleagues (2001b: pg 355) noted, "Generally, amplification of only *short* DNA pieces is possible" (italics ours), without providing clarification or citation for what is defined as "short". In their recently published introductory textbook, Brown and Brown (2011), stated "With even the best preserved specimens it is rarely possible to obtain products

longer than 300bp" (pg 118) and "Amplification products greater than 500 bp should be rare or never obtained" (pg 144). This introductory textbook was intentionally written without in-line citations, thus it is difficult to know upon which empirical data these claims are based. Cooper and Poinar (2000: pg 1139) noted that "large 500- to 1,000 base pair products are unusual". Lastly citing Pääbo (1989), Handt and colleagues (1994), and Höss and colleagues (1996) as evidence, Willerslev and Cooper (2005: pg 5) claimed "most ancient specimens do not contain any amplifiable endogenous DNA, while those that do possess only fragments in the 100–500 bp size range." Interestingly, in neither the review by Handt and colleagues (1994) nor the study of Höss and colleagues (1996) do the authors discuss the expected size range of aDNA molecules.

Highlighting the variation of length estimates for typical DNA recovered from ancient samples is not to suggest that they are incorrect. Taking a consensus view from the papers cited above, perhaps most aDNA molecules are less than 500 bps in length. However, given the paucity of cited empirical evidence, one can only conclude that this estimate has been drawn from personal experiences and/or intuition for what should be correct. The problem with this is that intuition should not be used as the underlying basis of a criterion for authenticating aDNA results. Many of the papers cited above draw evidence from a single study conducted twenty-three years ago by Pääbo (1989). In that study, whole genomic DNA extracted from 12 specimens, ranging in age from a four year old dried pork sample to a 13,000 year old ground sloth (*Mylodon sp.*) skin sample. After visualization of extracted DNA on an agarose gel (see Figure 1 in Pääbo, 1989) DNA fragment size was estimated to be on average 100-200 bps in length (with an estimated range of 40-500 bps). Given that recent whole genome extracts from ancient remains have been shown to contain large proportions of DNA not endogenous to the specimen (e.g. Green et al., 2006; Poinar et al., 2006), it is difficult to rely on estimates drawn from the agarose gel image of Pääbo (1989). It is fascinating to note that in only a *single specimen*, an ~4,000 year old liver sample from an Egyptian mummy, did Pääbo (1989) use PCR to estimate the intactness of the aDNA. In this case, the sample amplified in PCRs targeting 84 and 121 bp fragments of mtDNA, but not for 471 bps. These simple observations have largely formed our expectations for the state of aDNA in general.

With the adoption of next generation sequencing new estimations for the state of aDNA molecules have been made. For example, Briggs and colleagues (2009), found the mean length of Neanderthal mtDNA (from five specimens captured from library preparations) to range between 51.3-79.3 bps. From three of these same samples, Green and colleagues (2009) calculate that only 11-37% of the molecules are in excess of 80 bps. Mitochondrial DNA captured from the library preparations of 59 animal specimens (primates, horses, and cows), ranging from 18-2,400 years old, exhibited variable median lengths from 44-170 bps (Sawyer et al., 2012). The only samples with median lengths in excess of 100 bps were three monkey museum specimens that range 41-98 years old. Since intra-specimen ranges of DNA fragments were not reported, one can only conclude that 50% of the intact DNA in these samples was in excess of 44-170 bps in length. Combined with data from Briggs and colleagues (2009), median sizes of aDNA have been demonstrated to not be correlated with the age of the specimens, suggesting that degradation to short strand length occurs rapidly post-mortem (Sawyer et al., 2012). One possible explanation for this lack of correlation, which was not explored in either study, is that it might be an artifact originating from the way in which next generation sequencing libraries are produced. Short sections of DNA, containing sections for universal priming, are ligated onto blunt end repaired aDNA molecules, from

Comment [BK1]: Add later the new paper about the decay rate of aDNA (Allentoft et al 2012)

which PCR amplification is used to build the library. During the library build, theoretically all tagged molecules will be amplified. However, shorter molecules in the pool of template molecules will be preferentially amplified (Williams et al., 2006), possibly skewing the average molecule length to a lower end after the library build.

Other attempts to estimate the extent of aDNA fragmentation have circumvented this problem by directly estimating, via quantitative PCR (qPCR), the number of different sized fragments extracted from ancient remains. First, from a >20,000 year old Shasta ground sloth (*Nothrotheriops shastensis*) coprolite, Poinar and colleagues (2003) found that only 1% of the mtDNA fragments counted were ≥ 252 bps in length and 0.006% were ≥ 522 bps [7]. From the study of woolly mammoth (*Mammuthus primigenius*) bones from Siberia, Poinar and colleagues (2006) found that 45.5% of the mtDNA fragments counted were ≥151 bps in length and 0.3% were ≥921 bps. In a similar study of six mammoth remains, Schwarz and colleagues (2009), found that 19.2-51.1% of the counted mtDNA fragments from the EDTA supernatants (called SN fraction) and 51.5-75.8% from the dematerialized bone pellets (called PLT faction) were ≥151 bps. In one sample (BVM), fragments ≥921 bps comprised 0.9% of the molecules in the SN and 2.3% from the PLT. Of course, calculating portions of molecules in this manner is highly contingent on the size of the smallest fragment subjected to qPCR, and also the number of bins, but this latter observation would also be true of estimating the state of aDNA from library builds. Regardless, a qPCR approach to estimating the state of DNA recovered from samples has thus far been applied on samples that are unusually old, and most of which have been preserved under unusual conditions (i.e., permafrost preserved mammoths). This approach has not been explored across a wide range of samples.

Other recommendations for authenticating aDNA results rely on quantifying the number of template molecules that initiate a PCR. According to Willerslev and Cooper (2005: pg 7), this is "particularly important in studies where interpretation is based on few substitution differences, or few specimens." We believe that this recommendation is based on the idea that the number of staring template molecules should not be too large (which might indicate contamination) nor too small [which might permit miscoding lesions to be directly observed in the PCR product (see Figure 3 of Pääbo and colleagues (2004)]. There is also concern that when the starting number of aDNA molecules is very low, subsequent PCRs will be particularly prone to contamination (Bunce et al., 2012; Cooper and Poinar, 2000), which at times can completely outcompete the aDNA during amplification (e.g., Kemp et al., 2006; Kemp and Smith, 2005).

Nevertheless, what number or range of numbers is "just right" for aDNA templates has not been established, especially for the upper end. In other words—*Where does copy number of aDNA end and contaminating molecule copy number begin*? This is similar to debate in the forensic literature on what constitutes a LCN sample (Gill and Buckleton, 2010). On the lower end, however, Cooper and Poinar (2000: pg 1139) have stated "When the number of starting templates is low (<1,000), it may be impossible to exclude the possibility of sporadic contamination, especially for human DNA studies." Pääbo and colleagues (2004: pg 656), even argue that, "If consistent changes can be excluded (roughly for extracts containing >1000 template molecules), a single amplification is sufficient [for authentication of results]." This reference to 1000 molecules originates

[7] Assuming the qPCR numbers are additive, these figure is calculated as an average of 79 copies that are ≥252 bps and 0.5 copies ≥522 bps of the 7,653 copies that are ≤251 bps in length.

from a study conducted by Handt and colleagues (1996: pg 375) who stated "A minimum of 100-1,000 molecules per amplification may be necessary to obviate such problems [as those discussed above]". Their observation was made with cruder quantitative methods (i.e., competitive PCR) than have been developed since (i.e., qPCR), yet to our knowledge the relationship between starting template numbers and reliability of the resultant sequences have not been explored. Gilbert and colleagues (2003b: pg 43) more simply recommended that "…samples with low template numbers should be replicated…", but more recently Gilbert and colleagues (2009) have placed weight on replication over quantification in the authentication of aDNA results.

In any case, there are two obvious problems with the use of quantification in authenticating aDNA results. First, quantification alone cannot reveal if the pool of starting template molecules represents a heterogenous pool of authentic and contaminating molecules or a homogenous pool of either (Willerslev and Cooper, 2005). Secondly, as noted by Pääbo and colleagues (2004), if quantification of molecules is going to be properly used as a criterion of authentication, it must be conducted with every set of primer pairs, as the length of molecules in aDNA extracts is correlated with their copy number in a negative manner (Malmstrom et al., 2005). Likewise, quantification of a subset of samples should not be used to generalize about samples across an entire archaeological or paleontological site, as the quality and quantity of aDNA is probably sample specific (Winters et al., 2011), and indeed may vary within a single bone (Barta et al., In Review).

The goal of the present study is to follow up on related, but unpublished observations of post-mortem damage made by Kemp during a study of DNA extracted from an ~5,000 year old Native American skeleton from the Big Bar site (EhRk 4:1) in British Columbia (Malhi et al., 2007). During that study, over the standard procedure of amplifying and sequencing sections of hypervariable regions I and II (HVRI and II) ranging from 140-173 bps in length, Kemp attempted to amplify larger and larger fragments of the individual's mtDNA, which resulted in the recovery of amplicons as large as 859 bps in length (Table 1), an unexpected result given theoretical expectations for the degradation of DNA in solution (Pääbo and Wilson, 1991). The sequence of this amplicon exhibited transitions at nucleotide positions (nps) 16507 and 16508, mutations that were not identified in a smaller amplicon of 519 bps. Because: 1) the sequence of the 519 bp amplicon was consistent with sequences determined for smaller amplicons (Table 1), 2) the two mutational positions are not known to be variable among humans according to a search of mtDB - Human Mitochondrial Genome Database and Mitomap (Ruiz-Pesini et al., 2007), and 3) both mutations were C>T transitions [the most common form of miscoding lesions leads to artificial C>T "transitions" (Gilbert et al., 2007)], these observations are likely the product of post mortem genetic damage.

It is likely that most DNA extracted from a degraded specimen contains miscoding lesions, but these observations suggest that the ability to observe modified bases in direct sequences may be a function of the number of intact DNA molecules of a given length. This is at the root of concern over initiating a PCR from few template molecules, as discussed above. While not quantified in this case, it is hypothesized that damage was observed only in the largest amplicon because the frequency of template molecules of this size or larger must have been comparatively lower than those targeted by the other sets of primers. Since there were no observable underlying competitive peaks at nps 16507 and 16508, theoretically the PCR could have been initiated from a single molecule. Alternatively, this phenomenon of observing damage in direct sequences may

occur at smaller or larger amplicon sizes depending on the extent of post mortem strand-breakage, which is probably sample specific.

This hypothesis is explored by generating amplicons of variable length from ancient human samples from North and South America in order to determine the relationship between amplicon size and damage detection (here termed "Frequency Dependent Damage Detection" or FD^3). If a positive relationship is demonstrated, targeting long template molecules, even if they exist, may not be preferred when studying degraded samples. In additional, qPCR is employed to quantify starting template numbers in a number of these experiments. In total, this study assesses both appropriate molecular behavior and quantification as a means of authenticating aDNA results.

MATERIALS AND METHODS

Archaeological Samples

DNA extractions were performed on 127 ancient human samples and human bi-products (i.e., ancient feces or "coprolites") (Table 2). Site 42Bo1071 (N=1) is located near Willard Bay, Box Elder County, Utah, and dates to AD 1600-500 (Lambert and Simms, 2003). Aztec (ATB series, N=8) sacrificial victims excavated from Temple R in Tlatelolco date to AD 1457-57 and were previously studied by De la Cruz and colleagues (2008). Additional Post-Classic Aztecs (N=14) from Tlatelolco post-date AD 1325-1345 and were previously studied by Kemp and colleagues (2005). Human coprolites (BMII C series, N=20) from the Turkey Pen Ruins site (42SA3714 and 5109) located in Grand Gulch, San Juan County, southeast Utah, date to 200 BC to AD 450 (Speller et al., 2010). Cave 7 (site 42SA22180, N=2) in southeastern Utah dates to ca. 200 BC to AD 530 (Coltrain et al., 2012). Burials from CA-SCL-38 ("Yukisma", N=20) located in the Santa Clara Valley date Radiocarbon dating places deposition from 245 to at least 2205 YBP, with the majority being dated between 230-740 YBP (Gardner et al., 2011). Falls Creek Rock Shelter (FCRS and EBM samples, N=7) Falls Creek, CO. Kin Bineola (N=10) dates to ~1000 YBP. Fremont samples (NSRL series N=3) range from AD 779 to 1127. Peñasco Blanco (N=7) dates to ~1000 YBP. Pueblo Bonito (N=16) dates to ~1000 YBP. Talus Village Site (N=3) dates to ~2000 YBP. Colonial Maya samples (N=14) from Xcaret post-date 500 YBP. Two samples from Chile (Ayayene and Punta Santana) date to ~4500-6500 YBP.

DNA Methods

All preparation methods (i.e., extraction and PCR set-up) were conducted in the aDNA laboratory at Washington State University, one dedicated to the analysis of degraded and low copy number (LCN) DNA. Appropriate measures were employed to minimize contamination and, importantly, to detect it if present (Kemp and Smith, 2010).

DNA Extraction

Portions of the bones, teeth, and coprolites (i.e., desiccated feces) weighing between 26-641 mg (Table 2), were carefully removed from the whole. Bone and teeth samples were submerged in 6% w/v sodium hypochlorite (Clorox bleach) for four minutes to remove contaminating DNA from the surfaces of the samples (Kemp and Smith, 2005), followed by being rinsed twice with DNA-free ddH_2O. Coprolite material was not treated prior to extraction.

DNA was extracted from the samples in batches of seven (which is not reflected in Table 2, because some of the samples studied here were extracted with samples from other on-going studies in the Kemp lab), with an accompanying extraction negative control (labeled EC with the corresponding date of extraction in Table 2) using one or two of three methods (Table 2). First, some samples were extracted according to a modified protocol of Kemp and colleagues (2007) changed specifically in the silica extraction portion of the method as follows (labeled as Method #1 in Table 2): 1) following the isopropanol precipitation, 750 µL of 2% celite in 6M guanidine HCl[8] and 250 µL of 6M guanidine HCl were added to samples and vortexed numerous times over a 2 minute period, 2) 3 mL of DNA-free ddH_2O was pulled across the syringe and Promega Wizard® Minicolumns to wash them before pulling the samples across the columns, 3) the DNA bound silica was rinsed with 3 mL of 80% isopropanol (versus 2 mL recommended by the manufacturer), 4) 50 µL of 65ºC DNA-free ddH_2O was added to the column and left for 3 minutes prior to centrifugation, and this step was repeated again resulting in 100 µL of extracted DNA.

Second, some samples were extracted as just described, but an ethanol precipitation was conducted in place of the isopropanol precipitation described by Kemp and colleagues (2007) (labeled as Method #2 in Table 2). Following the phenol:chloroform extraction, one half volume of 5M ammonium acetate was added and to this combined volume was added 2 volumes of -20ºC absolute ethanol. The samples were inverted to mix this solution, and stored overnight at -20ºC.

For the third method, the samples were moved to 1.5 mL tubes to which 500 µL of EDTA (pH 8.0) was added (labeled as Method #3 in Table 2). The samples were incubated with agitation at room temperature for >48 hours. Three milligrams of proteinase K were added to the samples and incubated at 65ºC for 3 hours. The digested samples were extracted according to the modified silica extraction portion of the Kemp et al. (2007) method described above.

In total, the 127 ancient human bone, teeth, and coprolite samples were extracted in 26 batches (A-Z, Table 2) yielding 151 extracts.

Pre-Screening Samples for the Preservation of mtDNA (Haplogroup Determination) and Testing for Inhibitors

From batches A, B, and D (Table 2), the volumes of some extracts (i.e., CA-SCL-38 B4, CA-SCL-38 B80, CA-SCL-38 B171, BMII C3, BMII C7, Talus 3, and BMII C2) were split up and either diluted 1:10 or resilica extracted following the modified silica extraction portion of the Kemp et al. (2007) method just described. This produced an additional 6 extracts for a total of 157 processed as follows. All of the extracts were typed for the 9 bp deletion, which is one of the markers defining Native American mitochondrial haplogroup B, following Kemp and colleagues (2007). This amplicon is 112 bp if deleted and 121 bp if not deleted. A positive control, added in the post-PCR lab just prior to

[8] This solution is intended to mimic the Wizard® PCR Preps DNA Purification Resin, as best could be ascertained from the *Material Safety Data Sheet* (*MSDS*). To make this solution, add DNA-free ddH_2O to 1.25 mg of Celite® Analytical Filter Aid II (CAFA II, Sigma) up to 25mL, vortex and let incubate at room temperature overnight. Pour off the water carefully as to not pour off the celite and add DNA-free ddH_2O to 5mL and 6M guanidine HCl (Teknova) to 50mL.

running the PCRs, accompanied each batch of reactions to preclude PCR failure. The extraction negative controls and PCR negatives were tested in parallel with all rounds of amplification.

Extracts that produced negative results were tested for inhibitory effects following the rationale provided by Kemp and colleagues (2006) and as recently put into practice by Grier and colleagues (2013) using DNA extracted from ~170-415 year old goose remains (Wilson et al., 2011) as the "positive aDNA controls." Extraction negative controls were also all tested for the presence of inhibitors.

Fifteen microliter PCRs, which included 1.5 μL of ancient goose positive contol, were set up according to amplify a 159 bp portion of goose mitochondrial cytochrome B gene using the primers "BSP-I" and "GooseR" described by Wilson and colleagues (2011). To these reactions, 1.5 μL of the ancient human DNA extract or extraction negative control was added (totaling 16.5 μL reactions). PCRs were run according to Wilson and colleagues (2011) in parallel with a goose PCR that was not spiked (this reaction was used as a positive control, which allowed us to preclude PCR failure from contributing to our results [(Kemp and Smith, 2010)]. PCR negatives accompanied each round of amplification. If the goose DNA failed to amplify when spiked with ancient human DNA extract or extraction negative control, we concluded them to be inhibited. If the goose DNA amplified when spiked, but exhibited a noticeably dimmer band, we considered this to be "slightly" inhibited, and treated it as if it were inhibited.

Extracts deemed to be inhibited were subjected to "repeat silica extraction" (Kemp et al., 2006) following the modified silica extraction portion of the Kemp et al. (2007) method just described, adjusting for expended volume. Following repeated silica clean up, extractions were again screened for the 9 bp deletion. Samples that failed to amplify were tested again for inhibition and repeat silica extracted if necessary. All samples extracted in this study were carried to the point at which they: 1) yielded negative results and were deemed "free" of inhibitors, or 2) yielded positive amplification in screening for the 9 bp deletion and were deemed "free" of inhibitors as determined with the methodology just described. In the case of the former scenario, samples were concluded to not contain analyzable mtDNA and were no longer processed in the following experiments. In the case of the latter scenario, the samples were subsequently screened for the markers defining Native American mitochondrial haplogroups A, C, and D following Kemp and colleagues (2007). These amplicons range from 113 bp (haplogroup D) to 176 bp (haplogroup A) and 183 bp (haplogroup C). All extracts that were determined to contain a Native American mtDNA type, even if there was observable non-Native American mtDNA among the amplicons (e.g., in an incomplete digestion of the haplogroup A fragment when subjected with *HaeIII*), were considered in the experiments describe below. Ancient coprolite samples can also potentially contain more than one Native American DNA type due to leaching as result of depositional site processes. Observation of contamination in a sample might indicate that the extraction itself is contaminated or it could have originated in the PCR from which it was observed. This allowed us to analyze both the behavior of the aDNA and, in the case of a contaminated sample, the behavior of both the aDNA and contaminant DNA.

Testing Samples for the Preservation of Increasingly Long mtDNA Fragments I

A portion of the extractions (i.e., those from Batches A-G, Table 2) determined to contain Native American mtDNA were subjected to the following experiments. All

corresponding extraction negative controls from these batches were included in these experiments. Extracts were amplified for eleven fragment lengths ranging from 149-1144 bps across the d-loop, using a common forward primer (i.e., 15986F) and eleven reverse primers (Table 3). This approach is depicted in figure 1A and where it is contrasted against the more conservative approach for sequencing the d-loop in eleven short overlapping fragments (figure 1B) as employed by Kemp and colleagues (2007).

Fifteen microliter PCRs contained 3.2 mM dNTPs, 1 X PCR Buffer (Invitogen), 1.5 mM $MgCl_2$, 240 nM of each primer, and 0.3 U of Platinum® Taq DNA Polymerase. PCR conditions were as follows: 94°C for 3 min, followed by sixty cycles of denaturing at 94°C of 15 sec, annealing (according to Table 3, minus 0.1°C each round) for 15 sec, and extension at 72°C for 30 sec, followed by a final 72°C extension for 3 min. PCR negatives were run in parallel with each batch of PCRs. One or two positive controls (DNA extract from a buccal swab), added in the post-PCR lab just prior to running the PCRs, accompanied each batch of reactions to preclude PCR failure.

PCR products were run on 2% agarose gels stained with ethidium bromide to confirm amplification. All amplicons (including contamination, but not the positive controls) were prepared for sequencing by addition of 10 U of *ExoI* and 2 U of FastAP. Reactions were incubated at 37º C for 20 min, followed by 80ºC for 20 min. Sequences were generated in both directions at Elim Biopharm (Hayward, CA) aligned against the Cambridge Reference Sequence (Anderson et al., 1981; Andrews et al., 1999) in Sequencher (version 4.8).

Testing Samples for the Preservation of Increasingly Long mtDNA Fragments II

The remaining portion of the extractions (batches H-Z, Table 2) determined to contain analyzable mtDNA were subjected to the following experiments. Extracts were amplified for nine fragment lengths ranging from 166-940 bps across the d-loop, using a common forward primer (i.e., 16190F) and nine reverse primers (Table 4). The choice to switch to this approach was done on account of Native American mtDNA haplogroups exhibiting characteristic mutations in the "D-loop 3" amplicon (Table 4). Thus, starting with this amplicon (versus "D-loop 1" in Table 3) makes these results easier to authenticate as endogenous to the samples. Amplifications from a number of samples were replicated for D-loop 3, 3-4, 3-5, 3-6, 3-7. After this experimental design was established and the experiments conducted, inclusion of an additional fragment 62 bp in length (i.e., D-loop 3* in Table 4) was included in the study [However, it should be noted that the results for the smallest fragment as of yet does not have corresponding sequence data, as the small amplicon length precludes traditional/commonly used sequencing chemistry and will require differing techniques in order to acquire these data].

Fifteen microliter qPCRs contained contained 3.0 mM dNTPs, 1 X PCR Buffer (Invitogen), 2.25 mM $MgCl_2$, 0.12X SYBR Green I, 1X ROX, 240 nM of each primer, and 0.3 U of Platinum® Taq DNA Polymerase. Quantitative PCR conditions were as follows: 94°C for 3 min, followed by sixty cycles of denaturing at 94°C of 15 sec, annealing for 30 sec, and extension at 72°C for 30 sec. Three no template controls (NTC) controls were run with each plate. Standard curves were run using eight dilutions of each respective target fragments (i.e., copy numbers of 10,000, 5000, 2500,1250, 625,125, 25, 5).

However, lower quantity standards (i.e., copy numbers of 5 and 25) were stochastic and may have been undetermined. Melt curve analysis was performed with every run and qPCR efficiency was between 87-100% with R^2 values > 0.990.

It was originally our plan to be able to quantify starting template number in each of ten different reactions [i.e., to make the data comparable to that of Poinar and colleagues (Poinar et al., 2006) and Schwarz and colleagues (2009), who quantified mammoth mtDNA fragments ranging from 84-921 bps]. However, only for the four shortest amplicons (i.e., D-loop 3*, D-loop 3, D-loop 3-4, and D-loop 3-5) were the counts found to be consistent and demonstrated efficiencies between 87-100%.

PCR products from reactions D-loop 3-6, D-loop 3-7, D-loop 3-8, and D-loop 3-9 were run on 2% agarose gels stained with ethidium bromide to confirm amplification. Amplicons were prepared for sequencing by addition of 10 U of *ExoI* and 2 U of FastAP (Fermentas). Reactions were incubated at 37º C for 20 min, followed by 80ºC for 20 min. Sequences were generated in both directions at Elim Biopharm (Hayward, CA) aligned against the Cambridge Reference Sequence (Anderson et al., 1981; Andrews et al., 1999) in Sequencher (version 4.8).

Miscoding Lesion Damage Analysis

In addition to screening damage to strand lengths as described above, we used the direct sequences of the amplicons to assess "mutations" that resulted from miscoding lesions. This was accomplished in a number of ways. First, we evaluated the sequences for markers that define Native American mtDNA haplogroups A-D, or additional expected markers given what is known about regional Native American diversity. For this, we relied on our database that currently contains 3806 partial or full d-loop sequences reported for Native Americans. This aided us in determining if the sequences are derived from the samples, and not originating from contamination. Secondly, for those samples that amplified for more than one of the fragments, we could directly compare the results of these sequences where they overlap. Inconsistencies here could be the result of damage. For those regions sequenced without overlap, we simply had to consider again what is known about Native American mtDNA diversity. Moreover we also relied on the web sites Mitomap (www.mitomap.org) (Ruiz-Pesini et al., 2007) and mtDB- Human Mitochondrial Genome Database (www.mtdb.igp.uu.se) (Ingman and Gyllensten, 2006), for mtDNA positions documented to be variable among humans in general.

RESULTS AND DISCUSSION

Testing Samples for the Preservation of Increasingly Long mtDNA Fragments I

Of the 42 extractions conducted in this part of the study (i.e., those from Batches A-G), 24 were typed as belonging to Native American mtDNA haplogroup A, B, C, or D (Table 2). An additional four extracts exhibited haplogroup B mtDNA mixed with non-haplogroup B mtDNA and one sample exhibited a mix for the haplogroup C marker. In total 28/42 samples (66.7%) were determined to contain Native American mtDNA, whether mixed or exhibiting a single type. The remaining 14 samples were demonstrated to not contain mtDNA or not to exhibit markers defining Native American mtDNA haplogroup A, B, C, or D. In a few cases, extraction negative controls amplified, but none were found to exhibit any these markers.

Considering the additional modified extractions from batches A, B, and D (i.e., CA-SCL-38 B4, CA-SCL-38 B80, CA-SCL-38 B171, BMII C3, BMII C7, Talus 3, and BMII C2), amplification of the eleven fragments (Table 3) was attempted on 36 extracts. Five extractions [FCRS 10 (RS) 5/17/11, BMII C7 5/17/11 (full), Talus 1 Powder 5/22/11 (full), BMII C4 5/25/11 (2RS), NSRL 1286 OLD 6/3/11 (2RS)] failed to amplify in any of these reactions. The remaining extractions produced a variable number of positive results, with successful amplifications ranging from 168-1020 bps (Table 5).

From these extracts, 89 amplifications were sequenced (Table 6). Probable contamination was observed in D-loop 1 of: 1) NSRL 1306 6/3/11 NEW (2RS) that contradicted the observations from the remaining amplicons from this extract as well as observations from NSRL 1306 OLD 6/3/11, and 2) BMII C7 5/17/11 (RS) that contradicted BMII C7 5/17/11 1:10. Contamination matching the d-loop sequence of Kemp was observed in three amplifications originating from two extractions: 1) D-loop 1-2 and 1-5 from Talus 3 5/22/11 (RS), and 2) D-loop 1-8 from Xcaret 94 OLD 6/3/11 (RS). Kemp's mtDNA also accounts for 4 of the 5 instances of contamination observed in the extraction negative controls. In the fifth incident, the D-loop 1 sequence of EC 6/3/11-2 matched the Cambridge Reference Sequence, a result that could not have originated from Kemp's mtDNA.

Damaged bases were observed in 13 of the 84 (15.5%) of the amplifications not compromised by contamination (Table 6). There is a trend towards observing damage in increasingly larger amplicons. In total, damage was observed in: 1) 16.7% (5/30) of D-loop 1 amplifications, 2) 20% (3/15) of D-loop 1-3 amplifications, 3) 14.3% (1/7) of D-loop 1-5 amplifications, 4) 60% (3/5) of D-loop 1-7 amplifications, and 5) 100% of the D-loop 1-10 amplifications (the sole amplification). D-loops 1-2, 1-4, and 1-6 amplifications exhibited no damage. Note that amplifications of D-loops 1-8 and 1-9 produced no positive results (Table 5).

Of the 8 extractions that amplified in two or more of the eleven reactions and exhibited damage, 5 of them did so only in the largest amplicons produced (Table 6). In the three samples that defied this pattern: 1) NSRL 1299 6/3/11 NEW (8RS) exhibited damage in D-loop 1, but not in D-loop 1-2, 2) Ayayene 6/8/11 exhibited damage in D-loop 1, but not in D-loop 1-2 and 2) CA-SCL-38 B 152 6/8/11 (2RS) exhibited damage in D-loop 1-3, but not in D-loop 1, 1-2, 1-5, or 1-6. Since we did not collect quantitative data in portion of the study, we can only suggest that deviations from the general pattern are due to stochasticity inherent when dealing with low copy number target DNA. In addition there were three extracts that amplified for only D-loop 1 and were also damaged: FCRS 9 (RS) 5/17/11, Talus 3 5/22/11 (full), 42 Bo1017 B10 6/8/11.

It is important to emphasize that most of the sequences (84.5%) did not exhibit damaged bases. On the larger end of these, 769 bp fragments from two different samples appear undamaged. Fragments without damaged bases were also observed ranging down the line from 690-168 bps. These observations demonstrate that aDNA can be very well preserved, with a wide range of variation in its state of length degradation.

Testing Samples for the Preservation of Increasingly Long mtDNA Fragments II

Of the 109 extractions conducted in this part of the study (i.e., those from Batches H-Z, Table 2), 70 were typed as belonging to Native American mtDNA haplogroup A, B, C, or

D (Table 2). An additional two extracts exhibited haplogroup B mtDNA mixed with non-haplogroup B mtDNA. In total 72/109 extracts (66%) were determined to contain Native American mtDNA, whether mixed or exhibiting a single type. The extraction from ATB 5 NEW 5/11/11 (RS) (from Batch C) was also re-analyzed in this portion of the study. Amplification for ten fragments (Table 4) from these extracts produced a variable number of positive results, with successful amplifications ranging from 62-736 bps (Table 7). The sequencing results from these amplifications are reported in Table 8

The percent of amplification of endogenous mtDNA, not compromised by contamination, across the ten PCRs (i.e., D-loop 3* to 3-11) decreases with fragment size: 1) 89% for D-loop 3* [Note that as these are still to be sequenced, this percentage could decrease with the removal of compromised sequences], 2) 56% for D-loop 3, 3) 32% for D-loop 3-4, 4) 22% for D-loop 3-5, 5) 9% for D-loop 3-6, and 6) 9% for D-loop 3-7. No amplicons were produced in the reactions for D-loop 3-8, 3-9, 3-10, 3-11 (Table 9). These figures exclude extraction negative controls, and further excludes unclear amplifications, specifically: 1) those that had no band on an agarose gel but did have a count/quantity from qPCR, 2) those that had low melt curve with a count/quantity and no visible gel band, or 3) a gel band but no count/quantity (Tables 7 and 8)

Damaged bases were observed in 21 of the 137 (15.3%) of the amplifications not compromised by contamination. There is a trend towards observing damage in increasingly larger amplicons (Tables 8 and 9). In total, damage was observed in: 1) 11.6% (8/69) of D-loop 3 amplifications, 2) 9.7% (3/31) of D-loop 3-4 amplifications, 3) 14.3% (3/21) of D-loop 3-5 amplifications, 4) 25% (2/8) of D-loop 3-6 amplifications, and 5) 62.5% (5/8) of the D-loop 3-7 amplifications. Of the 8 extractions that produced sequence in two or more of the eleven reactions and exhibited damage, 7 of them did so only in the largest amplicons produced (87.5%). In the four samples that defied this pattern: 1) Aztec 39 8/14/11 (full) exhibited damage in D-loop 3, but not in D-loop 3-4, 2) CA-SCL-38 B 25 (2RS) exhibited damage in D-loop 3 and 3-6, but not in D-loop 3-4, 3) ATB 10 (2RS) exhibited damage in D-loop 3 and D-loop 3-4, 4) BMII C21 12/1/11 (full) exhibited damages in all fragments except one replicate of D-loop 3. In addition there were three extracts that amplified for only D-loop 3 and were also damaged: Aztec 08 EtOH (4RS), Aztec 42 8/14/11 (2RS), Penasco Blanco 07 (full), Pueblo Bonito 14-NEW (2RS).

Results from some of the experiments were influenced by non-specific amplification, primer dimer (i.e., detected in low melt curves), or being of such low concentrations that sequencing of the amplicon was unachievable.

The majority of contamination witnessed in this portion of the study, as was true in the first portion, was attributable to Kemp (16256T; 16362C; 16519C, reative to the Cambridge Reference Sequence), with lesser frequencies attributable to individuals matching the Cambridge Reference Sequence (CRS) or belonging to haplogroups H5, H6, or L2a[9] (Table 8).

The frequency of amplification of extraction controls is negatively correlated with fragment length (Table 9). Percent contamination ranged from: 1) 80% (12/15) for D-

[9] Note that Monroe conducted all of the qPCR and she belongs to haplogroup V, exhibiting the following mutations compared to the CRS: 16298C;16311C;72G. H5, H6, and L2a is also not found among laboratory personnel.

loop 3* [these have yet to be sequenced], 2) 44% (11/25) for D-loop 3 (but, only 5 were sequencable), 3) 4.7% (1/21) for D-loop 3-4, 4) 4.7% (1/21) for D-loop 3-5, and 5) 4.7% (1/21) for D-loop 3-6. No extraction negative control amplified in the reactions of D-loop 3-7, 3-8, 3-9, 3-10, or 3-11. These observations contradict predictions that contamination should be better preserved than aDNA with regards to stand length.

Observed contamination within sample extracts, mixed with endogenous aDNA or observed alone, is reported in Tables 8 and 9. In contrast to contamination observed in the extraction negative controls, the percent contamination per instance of amplification increased with fragment lengths up to 486 bp (i.e., up to D-loop 3-6) followed by a decrease: 1) 21.6% (19/88) for D-loop 3, 1) 24.4% (10/41) for D-loop 3-4, 3) 27.6% (8/29) for D-loop 3-5, 4) 20% (2/10) for D-loop 3-6, 5) 11.1% (1/9) for D-loop 3-7, and 6) 1.3% (1/78) for D-loop 3-9. Contamination was not observed in D-loops 3-8, 3-10, or 3-11.

When contamination was observed, it was most often represented by a single haplotype present in any given amplicon; however the contaminating haplotype was not always repeatable (Table 8). In other cases, endogenous aDNA appears to be in competition with contaminating haplotypes, where one or the other or both are observed in a single amplicon. When contamination was found in conjunction with the endogenous haplotype a higher frequency was associated with damage.

Interestingly, two coprolite samples BMII C25 12/1/11 (RS) and BMII C28 12/1/11 (RS) each displayed two different Native American haplotypes (Table 8). In the case of sample BMII C25 12/1/11 (RS), it was originally typed has haplogroup C (based on the *Alu*I site gain at np 13262), however the sequences from different amplicons were of haplogroups C and B. Interestingly, the smaller amplicon (D-loop 3-4) produced a haplogroup C sequence; while a larger fragment size (D-loop 3-7) produced a haplogroup B sequence. In the case of sample BMII C28 12/1/11 (RS), it was originally typed has haplogroup B (based on the presence of the 9 bp deletion), however the sequences from different amplifications of D-loop 3 were of haplogroups B or C. Previous study of this sample also indicated that it contains mtDNA belonging to both haplogroups B and C (Kemp et al., 2010). Observing mixed types in ancient feces, while rare in our study, was not entirely unexpected due to leaching and site processes, as well as their deposition within a communal setting (latrine/midden area) (Gilbert et al., 2008; Gilbert et al., 2009; Poinar et al., 2009).

Quantitative PCR and DNA Preservation

To explore the relationship between template DNA copy number and amplicon length, only results with >0.01 copies/μL were considered, including amplifications from which we were unable to retrieve sequence data, as long as endogenous sequences were found in a majority of total amplicons. Results from amplifications that reveal complete contamination were excluded, with mixed samples not removed from this analysis. Since sequence data is presently unavailable for D-loop 3*, we included the qPCR data only if there was at least one other endogenous sequence from other fragments. Overall, the data collected in this study indicate that both mean and median copy numbers decrease with fragment size: 1) D-loop 3* 554.72 copies/μL (SD 1156), 2) D-loop 3 62.04 copies/μL (SD 139.3), 3) D-loop 3-4 13.07 copies/μL (SD 29.27), and D-loop 3-5 4.85 copies/μL (6.89) (Table 10). These observations are consistent with the

classic observation of a negative relationship between amplicon length and PCR efficiency (Pääbo, 1989; Pääbo et al., 1988), of which starting template copy number is one factor. Moreover, the pattern of these qPCR data is consistent with the findings of previous studies (Poinar et al., 2003; Poinar et al., 2006; Schwarz et al., 2009), despite our inability to confidently quantify amplicons ≥486 bps in length.

To explore the relationship between template DNA copy number and observable damage in the direct sequences, only non-contamination results with >0.01 copies/μL were considered. For D-loop 3, the 47 undamaged sequences originated from an average of 80.49 copies/μL (SD 164.32, range 0.36-1000.57) and the 8 damage sequences originated from an average of 12.34 copies/μL (SD 18.35, range 2.07-56.87) (Table 10). A two-tailed t-test demonstrated that these averages are not statistically distinguishable at the 0.05 level of probability (p=0.25). For D-loop 3-4, the 24 undamaged sequences originated from an average of 16.46 copies/μL (SD 33.29, range 0.046-159.34) and the 3 damage sequences originated from an average of 2.63 copies/μL (SD 3.83, range 0.01-7.03). A two-tailed t-test demonstrated that these averages are also not statistically distinguishable (p=0.486). For D-loop 3-5, the 15 undamaged sequences originated from an average of 4.83 copies/μL (SD 6.88, range 0.06-25.5). For this amplicon, both damaged sequences originated from <0.01 copies/μL, so no statistical test was performed.

CONCLUSIONS

Whereas the observation of damage across the amplicons sequenced in this study was in the minority (34/211 amplicons not compromised by contamination, 16.1%), a positive relationship between amplicon length and the occurrence of damage has been demonstrated. While we were only able to generate quantitative data for a few of these amplicons, it is justifiable to assume that our ever increasingly larger fragments were initiated from fewer and fewer molecules [based on the data presented here in conjunction with those from previous studies (e.g., Briggs et al., 2009; Pääbo, 1989; Pääbo et al., 1988; Poinar et al., 2003; Poinar et al., 2006; Sawyer et al., 2012; Schwarz et al., 2009)]. Thus, frequency dependent damage detection (or FD^3) is a characteristic of aDNA and may become useful in the authentication of future aDNA results. On the other hand, because of FD^3, targeting long template molecules, even if they exist, may not be preferred when studying degraded samples. As a complicating factor, it is curious that in the extracts that were quantified, there was no statistical difference in mean starting copy number for sequences that exhibited damage versus those that did not display damage. In addition, we note that none of our PCRs were initiated from >1000 molecules, demonstrating that this cut-off (Handt et al., 1996), while regularly cited (e.g., Cooper and Poinar, 2000; Pääbo et al., 2004), is arbitrary for generating authentic aDNA results.

Of the criteria used to authenticate aDNA results, “appropriate molecular behavior” of aDNA molecules is largely recognized as key. While many argue that most aDNA is degraded to perhaps under 500 bps in length (as reviewed in the introduction), the extent of its degradation with regards to strand length has previously only been systematically explored in exceptional samples (e.g., Neanderthals, Mammoths, Shasta ground sloth). While in this study we found that most aDNA molecules are indeed degraded below 500 bps in length, we have cases of authentic aDNA that in excess of this mark. In fact, one sample produced a 1020 bp amplicon, albeit one that showed

damage in the direct sequence that otherwise appears authentic. This observation, combined with the observations of undamaged fragments 690 bps in length and the unpublished results of the study by Kemp (detailed in the introduction) warrants caution in placing a definitive upper bounds for just how intact are aDNA molecules. Moreover, we found the contamination in this study to be both more degraded with regards to strand length and lower in copy number than some aDNA samples. Thus, in some cases, the observed characteristics of aDNA and contamination might be the reverse of widely held expectations.

The degree of post-mortem damage appears to sample specific, making it difficult to generalize for all aDNA specimens as a means for absolute authentication. We suggest that rather than turning to immediate suspicion of results like these from ancient samples, simply because they are “unusual”, replication of such results and demonstrating that the data makes sense should trump intuition when evaluating aDNA data.

ACKNOWLEDGMENTS

This project was supported by Award No. 2008-DN-BX-K008 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication/program/exhibition are those of the author(s) and do not necessarily reflect those of the Department of Justice. Thanks to Elissa Bullion for assistance in the laboratory. Thanks also to our collaborators for providing the ancient DNA samples: Rosemary Cambra, Steven LeBlanc, Alan Leventhal, Bill Lipe, RG Matson, Juan Alberto Roman Berrelleza, Steve Simms, and Alfonso Torre Blanco.

WORKS CITED

Alaeddini R (2011) Forensic implications of PCR inhibition--A review. Forensic Sci Int Genet 6:297-305.

Alaeddini R, Walsh SJ, and Abbas A (2010) Forensic implications of genetic analyses from degraded DNA--a review. Forensic Sci Int Genet 4:148-57.

Alonso A, Martin P, Albarran C, Garcia P, Garcia O, de Simon LF, Garcia-Hirschfeld J, Sancho M, de la Rua C, and Fernandez-Piqueras J (2004) Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. Forensic Science International 139:141-149.

Anderson S, Bankier AT, Barrel BG, DeBulin MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, and Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457-465.

Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, and Howell N (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nature Genetics 23:147.

Axelsson E, Willerslev E, Gilbert MT, and Nielsen R (2008) The effect of ancient DNA damage on inferences of demographic histories. Mol Biol Evol 25:2181-7.

Bär W, Brinkmann B, Budowle B, Carracedo A, Gill P, Holland M, Lincoln PJ, Mayr W, Morling N, Olaisen B, Schneider PM, Tully G, and Wilson M (2000) Guidelines for mitochondrial DNA typing. Vox Sanguinis 79:121-125.

Barta JL, Monroe C, and Kemp BM (In Review) Mitochondrial DNA Preservation Across Individual Bones is Not Related to Bone Density. Forensic Science International: Genetics.

Briggs AW, Good JM, Green RE, Krause J, Maricic T, Stenzel U, Lalueza-Fox C, Rudan P, Brajkovic D, Kucan Z, Gusic I, Schmitz R, Doronichev VB, Golovanova LV, de la Rasilla M, Fortea J, Rosas A, and Paabo S (2009) Targeted retrieval and analysis of five Neandertal mtDNA genomes. Science 325:318-21.

Brown T, and Brown K (2011) Biomolecular Archaeology: An Introduction. West Sussex, UK: Wiley-Blackwell.

Buckleton J, Walsh S, and Harbinson S (2005) Nonautosomal Forensic Markers. In J Buckleton, CM Triggs and S Walsh (eds.): Forensic DNA Evidence Interpretation. New York: CRC Press, pp. 299-340.

Bunce M, Oskam C, and Allentoft M (2012) Quantitative real-time PCR in aDNA research. In B Shapiro and M Hofreiter (eds.): Ancient DNA : Methods and Protocols. New York: Humana Press, pp. 121-132.

Capelli C, Tschentscher F, and Pascali VL (2003) "Ancient" protocols for the crime scene? Similarities and differences between forensic genetics and ancient DNA analysis. Forensic Science International 131:59-64.

Caramelli D, Lalueza-Fox C, Vernesi C, Lari M, Casoli A, Mallegni F, Chiarelli B, Dupanloup I, Bertranpetit J, Barbujani G, and Bertorelle G (2003) Evidence for a genetic discontinuity between Neandertals and 24,000-year-old anatomically modern Europeans. Proc Natl Acad Sci U S A 100:6593-7.

Caramelli D, Milani L, Vai S, Modi A, Pecchioli E, Girardi M, Pilli E, Lari M, Lippi B, Ronchitelli A, Mallegni F, Casoli A, Bertorelle G, and Barbujani G (2008) A 28,000 years old Cro-Magnon mtDNA sequence differs from all potentially contaminating modern sequences. PLoS One 3:e2700.

Coltrain JBC, Janetski JC, and Lewis MD (2012) A re-assessment of Basketmaker II cave7: massacre site or cemetery context. Journal of Archaeological Science 39:2220-2230.

Cooper A, and Poinar HN (2000) Ancient DNA: do it right or not at all. Science 289:1139.
De la Cruz I, González-Oliver A, Kemp BM, Román JA, Smith DG, and Torre-Blanco A (2008) Sex identification of infants sacrificed to the ancient Aztec raingods in Tlatelolco, Mexico. Current Anthropology 49:519-526.
Deagle BE, Eveson JP, and Jarman SN (2006) Quantification of damage in DNA recovered from highly degraded samples--a case study on DNA in faeces. Front Zool 3:11.
Edson SM, Ross JP, Coble MD, Parsons TJ, and Barritt SM (2004) Naming the Dead-Confronting the Realities of Rapid Identification of Degraded Skeletal Remains. Forensic Science Review 16:64-90.
Fattorini P, Ciofuli R, Cossutta F, Giulianini P, Edomi P, Furlanut M, and Previdere C (1999) Fidelity of polymerase chain reaction-direct sequencing analysis of damaged forensic samples. Electrophoresis 20:3349-57.
Fattorini P, Cossutta F, Giulianini P, Edomi P, and Previdere C (2000) DNA damage promotes mistyping in the allele specific oligonucleotide probing analysis of forensic samples. Electrophoresis 21:2969-72.
Gardner KS, Leventhal A, Cambra R, Tibe MO, Bartelink EJ, and Martinez A (2011) Mothers and infants in the prehistoric Santa Clara Valley: What stable isotpoes tell us about ancestral Ohlone weaning practices: 25th annual meeting of the Society for California Archaeology.
Gilbert MT, Binladen J, Miller W, Wiuf C, Willerslev E, Poinar H, Carlson JE, Leebens-Mack JH, and Schuster SC (2007) Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis. Nucleic Acids Res 35:1-10.
Gilbert MT, Hansen AJ, Willerslev E, Rudbeck L, Barnes I, Lynnerup N, and Cooper A (2003a) Characterization of genetic miscoding lesions caused by postmortem damage. Am J Hum Genet 72:48-61.
Gilbert MT, Willerslev E, Hansen AJ, Barnes I, Rudbeck L, Lynnerup N, and Cooper A (2003b) Distribution patterns of postmortem damage in human mitochondrial DNA. Am J Hum Genet 72:32-47.
Gilbert MTP (2006) Postmortem Damage of Mitochondrial DNA. In H-J Bandelt, V Macaulay and M Richards (eds.): Human Mitochondrial DNA and the Evolution of Homo sapiens. Berlin: Springer-Verlag pp. 91-115.
Gilbert MTP, Bandelt H-J, Hofreiter M, and Barnes I (2005) Assessing ancient DNA studies. TRENDS in Ecology and Evolution 20:541-544.
Gilbert MTP, Jenkins DL, Gotherstrom A, Naveran N, Sanchez JJ, Hofreiter M, Thomsen PF, Binladen J, Higham TFG, Yohe RM, II, Parr R, Cummings LS, and Willerslev E (2008) DNA from pre-Clovis human coprolites in Oregon, North America. Science 320:786-9.
Gilbert MTP, Jenkins DL, Higham TFG, Rasmussen M, Malmstrom H, Svensson EM, Sanchez JJ, Cummings LS, Yohe RM, II, Hofreiter M, Gotherstrom A, and Willerslev E (2009) Response to Comment by Poinar et al. on "DNA from Pre-Clovis Human Coprolites in Oregon, North America". Science 325:148-b.
Gill P, and Buckleton J (2010) A universal strategy to interpret DNA profiles that does not require a definition of low-copy-number. Forensic Sci Int Genet 4:221-7.
Green RE, Briggs AW, Krause J, Prufer K, Burbano HA, Siebauer M, Lachmann M, and Paabo S (2009) The Neandertal genome and ancient DNA authenticity. EMBO J 28:2494-502.
Green RE, Krause J, Ptak SE, Briggs AW, Ronan MT, Simons JF, Du L, Egholm M, Rothberg JM, Paunovic M, and Paabo S (2006) Analysis of one million base pairs of Neanderthal DNA. Nature 444:330-6.

Grier C, Flanigan K, Winters M, Jordan LG, Lukowski S, and Kemp BM (2013) Using Ancient DNA Identification and Osteometric Measures of Archaeological Pacific Salmon Vertebrae for Reconstructing Salmon Fisheries and Site Seasonality at Dionisio Point, British Columbia. Journal of Archaeological Science 40:544-555.
Handt O, Hoss M, Krings M, and Paabo S (1994) Ancient DNA: methodological challenges. Experientia 50:524-9.
Handt O, Krings M, Ward RH, and Paabo S (1996) The retrieval of ancient human DNA sequences. American Journal of Human Genetics 59:368-376.
Hekkala E, Shirley MH, Amato G, Austin JD, Charter S, Thorbjarnarson J, Vliet KA, Houck ML, Desalle R, and Blum MJ (2011) An ancient icon reveals new mysteries: mummy DNA resurrects a cryptic species within the Nile crocodile. Mol Ecol.
Ho SY, Heupink TH, Rambaut A, and Shapiro B (2007a) Bayesian estimation of sequence damage in ancient DNA. Mol Biol Evol 24:1416-22.
Ho SY, Kolokotronis SO, and Allaby RG (2007b) Elevated substitution rates estimated from ancient DNA sequences. Biol Lett 3:702-5.
Hofreiter M, Jaenicke V, Serre D, von Haeseler A, and Pääbo S (2001a) DNA sequence from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. Nucleic Acids Research 29:4793-4799.
Hofreiter M, Serre D, Poinar HN, Kuch M, and Paabo S (2001b) Ancient DNA. Nat Rev Genet 2:353-9.
Höss M, Jaruga P, Zastawny TH, Dizdaroglu M, and Pääbo S (1996) DNA damage and DNA sequence retrieval from ancient tissues. Nucleic Acids Res 24:1304-7.
Ingman M, and Gyllensten U (2006) mtDB: Human Mitochondrial Genome Database, a resource for population genetics and medical sciences. Nucleic Acids Res 34:D749-51.
Jobling MA, and Gill P (2004) Encoded evidence: DNA in forensic analysis. Nat Rev Genet 5:739-51.
Kemp BM, Malhi RS, McDonough J, Bolnick DA, Eshleman JA, Rickards O, Martinez-Labarga C, Johnson JR, Lorenz JG, Dixon EJ, Fifield TE, Heaton TH, Worl R, and Smith DG (2007) Genetic analysis of early Holocene skeletal remains from Alaska and its implication for the settlement of the Americas. American Journal of Physical Anthropology 132:605-621.
Kemp BM, Monroe C, Geib PR, Matson RG, and Lipe WD (2010) Genetic Analysis of Coprolites from Southeastern Utah. 75th Annual Meeting of the Society for American Archaeology.
Kemp BM, Monroe C, and Smith DG (2006) Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts. Journal of Archaeological Science 33:1680-1689.
Kemp BM, Resendez A, Román Berrelleza JA, Malhi RS, and Smith DG (2005) An Analysis of Ancient Aztec mtDNA from Tlatelolco: Pre-Columbian Relations and the Spread of Uto-Aztecan. In DM Reed (ed.): Biomolecular Archaeology: Genetic Approaches to the Past. Carbondale, IL: Southern Illinois University, pp. 22-46.
Kemp BM, and Smith DG (2005) Use of Bleach to Eliminate Contaminating DNA from the Surfaces of Bones and Teeth. Forensic Science International 154:53-61.
Kemp BM, and Smith DG (2010) Ancient DNA Methodology: "Thoughts from Brian M. Kemp and David Glenn Smith on "Mitochondrial DNA of Protohistoric Remains of an Arikara Population from South Dakota". Human Biology 82:227-238.

Lambert PM, and Simms SR (2003) Archaeological and Analysis of Prehistoric Human Remains Near Willard Bay, Utah. Utah State University Contributions to Anthropology No. 33. Logan, UT.
Lawrence DM, Kemp BM, Eshleman J, Jantz RL, Snow M, George D, and Smith DG (2010) Mitochondrial DNA of Protohistoric Remains of an Arikara Population from South Dakota (USA): Implications for the Macro-Siouan Language Hypothesis. Human Biology 82:157-178.
Malhi RS, Kemp BM, Eshleman JA, Cybulski J, Smith DG, Cousins S, and Harry H (2007) Haplogroup M Discovered in Prehistoric North America. Journal of Archaeological Science 34:642-648.
Malmstrom H, Gilbert MT, Thomas MG, Brandstrom M, Stora J, Molnar P, Andersen PK, Bendixen C, Holmlund G, Gotherstrom A, and Willerslev E (2009) Ancient DNA reveals lack of continuity between neolithic hunter-gatherers and contemporary Scandinavians. Curr Biol 19:1758-62.
Malmstrom H, Stora J, Dalen L, Holmlund G, and Gotherstrom A (2005) Extensive human DNA contamination in extracts from ancient dog bones and teeth. Mol Biol Evol 22:2040-7.
Malmstrom H, Svensson EM, Gilbert MT, Willerslev E, Gotherstrom A, and Holmlund G (2007) More on contamination: the use of asymmetric molecular behavior to identify authentic ancient human DNA. Mol Biol Evol 24:998-1004.
O'Rourke DH, Hayes MG, and Carlyle SW (2000) Ancient DNA studies in physical anthropology. Annual Review of Anthropology 29:217-242.
Ottoni C, Koon HE, Collins MJ, Penkman KE, Rickards O, and Craig OE (2009) Preservation of ancient DNA in thermally damaged archaeological bone. Naturwissenschaften 96:267-78.
Pääbo S (1989) Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proc Natl Acad Sci U S A 86:1939-43.
Pääbo S, Gifford JA, and Wilson AC (1988) Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Research 16:9775-9788.
Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, and Hofreiter M (2004) Genetic analyses from ancient DNA. Annu Rev Genet 38:645-79.
Pääbo S, and Wilson AC (1991) Miocene DNA sequences - a dream come true? Curr Biol 1:45-6.
Poinar H, Fiedel S, King CE, Devault AM, Bos K, Kuch M, and Debruyne R (2009) Comment on "DNA from pre-Clovis human coprolites in Oregon, North America". Science 325:148-a.
Poinar H, Kuch M, McDonald G, Martin P, and Paabo S (2003) Nuclear gene sequences from a late Pleistocene sloth coprolite. Current Biology 12:1150-1152.
Poinar HN, Schwarz C, Qi J, Shapiro B, Macphee RD, Buigues B, Tikhonov A, Huson DH, Tomsho LP, Auch A, Rampp M, Miller W, and Schuster SC (2006) Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA. Science 311:392-4.
Previdere C, Micheletti P, Perossa R, Grignani P, and Fattorini P (2002) Molecular characterisation of the nucleic acids recovered from aged forensic samples. Int J Legal Med 116:334-339.
Pusch CM, and Bachmann L (2004) Spiking of contemporary human template DNA with ancient DNA extracts induces mutations under PCR and generates nonauthentic mitochondrial sequences. Mol Biol Evol 21:957-64.

Rambaut A, Ho SY, Drummond AJ, and Shapiro B (2009) Accommodating the effect of ancient DNA damage on inferences of demographic histories. Mol Biol Evol 26:245-8.
Ruiz-Pesini E, Lott MT, Procaccio V, Poole JC, Brandon MC, Mishmar D, Yi C, Kreuziger J, Baldi P, and Wallace DC (2007) An enhanced MITOMAP with a global mtDNA mutational phylogeny. Nucleic Acids Res 35:D823-8.
Sawyer S, Krause J, Guschanski K, Savolainen V, and Paabo S (2012) Temporal patterns of nucleotide misincorporations and DNA fragmentation in ancient DNA. PloS One 7:e34131.
Schwarz C, Debruyne R, Kuch M, McNally E, Schwarcz H, Aubrey AD, Bada J, and Poinar H (2009) New insights from old bones: DNA preservation and degradation in permafrost preserved mammoth remains. Nucleic Acids Res 37:3215-29.
Speller CF, Kemp BM, Wyatt SD, Monroe C, Lipe WD, Arndt UM, and Yang DY (2010) Ancient Mitochondrial DNA Analysis Reveals Complexity of Indigenous North American Turkey Domestication. Proceedings of the National Academy of Science USA 107:2807-2812.
Tully G, Bar W, Brinkmann B, Carracedo A, Gill P, Morling N, Parson W, and Schneider PM (2001) Consideratons by the European DNA profiling (EDNAP) group on the working practices, nomeclature and interpretation of mitochondrial DNA profiles. Forensic Sci Int 124:83-91.
von Wurmb-Schwark N, Heinrich A, Freudenberg M, Gebuhr M, and Schwark T (2008) The impact of DNA contamination of bone samples in forensic case analysis and anthropological research. Legal Medicine 10:125-130.
Wang H, Ge B, Mair VH, Cai D, Xie C, Zhang Q, Zhou H, and Zhu H (2007) Molecular genetic analysis of remains from Lamadong cemetery, Liaoning, China. Am J Phys Anthropol 134:404-11.
Willerslev E, and Cooper A (2005) Ancient DNA. Proc Biol Sci 272:3-16.
Williams R, Peisajovich SG, Miller OJ, Magdassi S, Tawfik DS, and Griffiths AD (2006) Amplification of complex gene libraries by emulsion PCR. Nat Methods 3:545-50.
Wilson BW, Crockford SJ, Johnson JW, Malhi RS, and Kemp BM (2010) Genetic and archaeological evidence for a former breeding population of Aleutian cackling goose, Branta hutchinsii leucopareia, on Adak Island, central Aleutians, Alaska. Canadian Journal of Zooology:732-743.
Wilson BW, Crockford SJ, Johnson JW, Malhi RS, and Kemp BM (2011) Genetic and archaeological evidence for a former breeding population of Aleutian cackling goose, Branta hutchinsii leucopareia, on Adak Island, central Aleutians, Alaska. Canadian Journal of Zooology:732-743.
Winters M, Barta JL, Monroe C, and Kemp BM (2011) To clone or not to clone: Method analysis for retrieving consensus sequences in ancient DNA samples. PLoS One 6:e21247.
Yang DY, and Watt K (2005) Contamination controls when preparing archaeological remains for ancient DNA analysis. Journal of Archaeological Science 32:331-336.

Table 1. Unpublished observations of mtDNA sequencing conducted by Kemp during a study of DNA extracted from an ~5,000 year old Native American skeleton from British Columbia (Malhi et al., 2007). Primers used are described by Malhi and colleagues (2007) and Kemp and colleagues (2007). Bolded mutations are likely the result of miscoding lesions. Nucleotide positions are relative to the Cambridge Reference Sequence (Anderson et al., 1981; Andrews et al., 1999).

Primers	Amplicon Sizes (bps)	Sequence Spans (nps)	Sequence
Four sets, D-loop 1, 2, 3, and 4	Four overlapping fragements,140-147	16011-16382	16111T, 16223T, 16278T, 16290T, 16319A, 16362C
Three sets, D-loop 7, 8, 9	Three overlapping fragements,152-173	00059-00330	00064T, 00073G, 00146C, 00153G, 00235T, 00263G, 00309.1C, 00309.2C
15986-16355	370	16011-16330	16111T, 16223T, 16278T, 16290T, 16319A
15986-16404	419	16011-16382	16111T, 16223T, 16278T, 16290T, 16319A, 16362C
15986-16549	564	16011-16529	16111T, 16223T, 16278T, 16290T, 16319A, 16362C
15986-00275	859	16011-00248	16111T, 16223T, 16278T, 16290T, 16319A, 16362C, **16507T**, **16508T**, 00064T, 00073G, 00146C, 00153G, 00235T

Table 2. Samples included in this study, amount and type of material extracted, extraction method, number of re-silica extractions, modifications to the extracts, and haplogroup of the sample, if determined.

For ease of viewing this table, it has been included as an excel file called "Appendix F" with this NIJ final report.

Table 3. Fragments targeted under "*Testing Samples for the Preservation of Increasingly Long mtDNA Fragments I*", primers [as described by Kemp and colleagues (Kemp et al., 2007)], annealing temperatures for the various reaction, and amplicon sizes. Nucleotide positions are relative to the Cambridge Reference Sequence (Anderson et al., 1981; Andrews et al., 1999),

Target	Primers	Sequences Span (nps)	Annealing temperature	Amplicon Size (bps)
D-loop 1	15986-16153	16011-16131	62°C	168
D-loop 1-2	15986-16251	16011-16229	62°C	266
D-loop 1-3	15986-16355	16011-16330	58°C	370
D-loop 1-4	15986-16404	16011-16382	58°C	419
D-loop 1-5	15986-16549	16011-16529	62°C	564
D-loop 1-6	15986-00106	16011-00081	62°C	690
D-loop 1-7	15986-00185	16011-00159	62°C	769
D-loop 1-8	15986-00275	16011-00248	62°C	859
D-loop 1-9	15986-00356	16011-00330	62°C	940
D-loop 1-10	15986-00436	16011-00414	62°C	1020
D-loop 1-11	15986-00560	16011-00537	62°C	1144

Table 4. Fragments targeted under "*Quantitative PCR and Testing Samples for the Preservation of Increasingly Long mtDNA Fragments II*", primers [as described by Kemp and colleagues (Kemp et al., 2007)], annealing temperatures for the various reaction, and amplicon sizes. Nucleotide positions are relative to the Cambridge Reference Sequence (Anderson et al., 1981; Andrews et al., 1999),

Target	Primers	Sequences Span (nps)	Annealing temperature	Amplicon Size (bps)
D-loop 3*	16190-16251	16210-16229	60	62
D-loop 3	16190-16355	16210-16330	60	166
D-loop 3-4	16190-16404	16210-16382	60	215
D-loop 3-5	16190-16549	16210-16529	60	360
D-loop 3-6	16190-00106	16210-00081	60	486
D-loop 3-7	16190-00185	16210-00159	60	565
D-loop 3-8	16190-00275	16210-00248	60	655
D-loop 3-9	16190-00356	16210-00330	60	736
D-loop 3-10	16190-00436	16210-00414	60	816
D-loop 3-11	16190-00560	16210-00537	60	940

Table 5. Amplification results from "*Testing Samples for the Preservation of Increasingly Long mtDNA Fragments I*". Note that "weak" indications amplification, but the band on the gel appeared markedly dimmer than the other bands.

Table 6. Sequencing results from "*Testing Samples for the Preservation of Increasingly Long mtDNA Fragments I*". Mutations reported relative the Cambridge Reference Sequence (Anderson et al., 1981; Andrews et al., 1999). Bolded mutations are likely the product of post-mortem damage.

For ease of viewing tables 5 and 6, these has been included as an excel file called "Appendix G" with this NIJ final report.

Table 7. Amplification results from "*Testing Samples for the Preservation of Increasingly Long mtDNA Fragments II*". Key: O= no amplification; X/O= unclear amplification; X= amplification; X gray= contamination; X bold=damage; X bold gray= contamination with damage; x=amplification but no sequence data

Table 8. Sequencing results from "*Testing Samples for the Preservation of Increasingly Long mtDNA Fragments II*". Quantification is reported as copies/μL. Mutations reported relative the Cambridge Reference Sequence (Anderson et al., 1981; Andrews et al., 1999). Key: Mutations in bold= damage; Mutations in bold italics= damage as a transversion; Mutations in gray =contamination; Mutations in bold gray= contamination with damage

For ease of viewing tables 7 and 8, these has been included as an excel file called "Appendix H" with this NIJ final report.

Table 9. Summary of results from "*Testing Samples for the Preservation of Increasingly Long mtDNA Fragments II*", categorized and tallied according to whether the results were deemed: A) authentic, B) to have resulted from contamination, C) compromised by damage, and D) observed in the extraction negative controls.

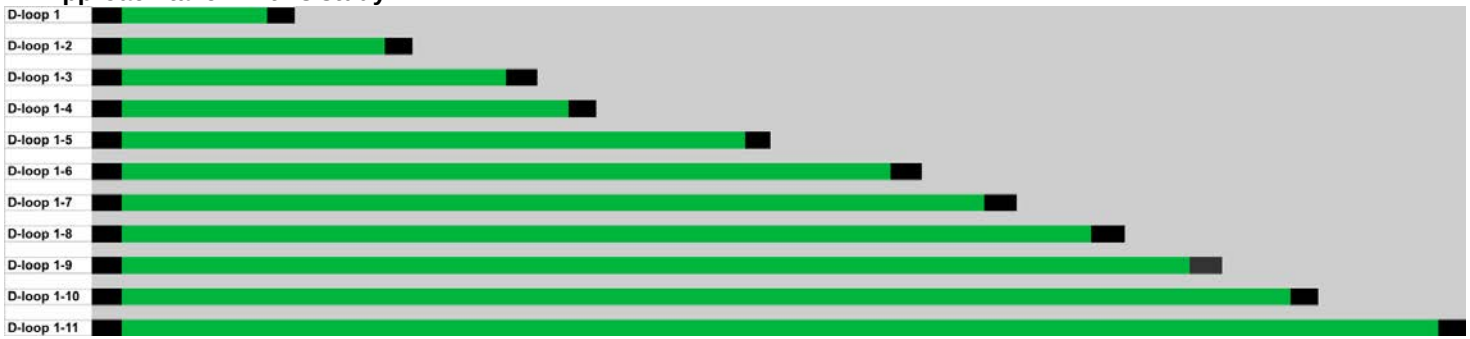
A. Authentic	D-loop 3*	Frequency	D-loop 3	Frequency	D-loop 3-4	Frequency	D-loop 3-5	Frequency	D-loop 3-6	Frequency	D-loop 3-7	D-loop 3-8	D-loop 3-9	D-loop 3-10	D-loop 3-11
Amplification total	65/73	0.890	88/125	0.704	41/97	0.423	29/94	0.309	10/94	0.106	9/93	0	0/78	0	0
Amplification total without contamination	N/A	N/A	70/125	0.560	31/97	0.320	21/94	0.223	8/94	0.085	8/93	0	0	0	0
unclear amplification/quantification (X/O)* total	0	0	10/125	0.080	2/97	0.021	0/94	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
B. Contamination															
Contamination total	N/A	N/A	19/88	0.216	10/41	0.244	8/29	0.276	2/10	0.200	1/9	0	1/78	0	0
Single contaminate only	N/A	N/A	12/18	0.667	6/10	0.600	6/8	0.750	2/10	0.200	1/9	0	1/78	0	0
Damage within only contamination	N/A	N/A	1/19	0.053											
Contamination-replication (not necessarily a repeat of contam)	N/A	N/A	5/52	0.096	1/17	0.059	0/16	0	0/16	0	0/93	0	0	0	0
Contamination- with endogenous type	N/A	N/A	2/18	0.111	1/10	0.100	0/29	0	0/10	0	0/93	0	0	0	0
Contamination- with endogenous type with damage	N/A	N/A	4/18	0.222	2/10	0.200	2/8	0.250	0/10	0	0/93	0	0	0	0
Damage freq per instance of amplfication of contamination	N/A	N/A	4/18	0.222	2/10	0.200	11/40	0.275	0	0	0	0	0.013	0	0
C. Damage															
Damage freq per instance of amplfication without contamination	N/A	N/A	8/69	0.116	3/31	0.096	3/21	0.143	2/8	0.250	5/8	0	0	0	0
Damage- total attempted amplifications	N/A	N/A	8/125	0.064	3/97	0.031	3/94	0.032	2/94	0.020	5/93	0	0	0	0
D. Extraction Negative Control															
Amplification total	12/15	0.80	11/25	0.440	1/21	0.048	1/21	0.048	1/21	0.048	0	0	0	0	0
Unclear amplification (X/O)*	N/A		3/25	0.120	2/21	0.095	1/21	0.048	0	0.000	0	0	0	0	0
Contamination-successful sequence	N/A		5/11	0.455	1/21	0.048	1/21	0.048	1/21	0.048	0	0	0	0	0
Damage	N/A		2/25	0.080	0	0	1/21	0.048	0	0	0	0	0	0	0

Table 10. Summary of qPCR data. Mean counts, standard deviation, median, minimum, maximum, and sample size for: A) All fragments, B) those fragments deemed to be without damage, and C) those fragments that exhibited damage.

A. All fragments	D-loop 3*	D-loop 3	D-loop 3-4	D-loop 3-5
Mean	554.72243	62.04275851	13.07150595	4.84907075
Std	1156.041237	139.3060298	29.27125584	6.885389417
Median	102.7063751	11.5886	3.73795	2.17222
Minimum	1.59909308	0.0376795	0.0109648	0.0302455
Maximum	6556.196289	1001.57	159.342	9.32251
N=	54	72	32	13
B. Fragments without damage				
Mean	n/a	80.49040168	16.46376081	4.829038873
Std	n/a	164.3222893	33.29075819	6.881555732
Median	n/a	18.8698	4.31966	1.89947
Minimum	n/a	0.361449	0.0465195	0.056804
Maximum	n/a	1001.57	159.342	25.5128
N=	n/a	47	24	15
C. Damaged fragments				
Mean	n/a	12.34425888	2.6281166	n/a
Std	n/a	18.35327249	3.834425422	n/a
Median	n/a	7.65739	0.843845	n/a
Minimum	n/a	2.07193	0.0109648	0.0109648
Maximum	n/a	56.8708	7.02954	0.00071339
N=	n/a	8	3	2

Figure 1A. Visual depiction of the experimental design described under "*Testing Samples for the Preservation of Increasingly Long mtDNA Fragments I*", Black sections represent primers described in Table Y and the green sections represent the remain portions of the amplicons. **Figure 1B.** This approach is contrasted against the more conservative approach, using the same primers, for sequencing the d-loop in eleven short overlapping fragments as employed by Kemp and colleagues (2007).

A. Approach taken in this study.



B. Approach taken by Kemp and colleagues (2007).

Note: This study has been previously published in *PLoS One*

Winters M, Barta JL, Monroe C, and Kemp BM (2011) To clone or not to clone: Method analysis for retrieving consensus sequences in ancient DNA samples. *PLoS One* 6:e21247.

To Clone or Not to Clone: Method Analysis for Retrieving Consensus Sequences in Ancient DNA Samples

Misa Winters[1], Jodi Lynn Barta[1,2], Cara Monroe[1,2,3], and Brian M. Kemp[1,2]

[1]School of Biological Sciences, Washington State University, Pullman, WA 99164-4236
[2]Department of Anthropology, Washington State University, Pullman, WA 99164-4910
[3]Department of Anthropology, University of California-Santa Barbara, Santa Barbara, CA 93106-3210

Corresponding author:

Brian M. Kemp
Department of Anthropology
Washington State University
Pullman, WA 99164-4910
Office: 509.335.7403
Fax: 509.335.3999
e-mail:bmkemp@wsu.edu

Key words**: Ancient DNA, Cloning, mtDNA, Postmortem Damage**

Data deposition footnote: Mitochondrial DNA sequences have been submitted to Genbank and assigned accession numbers HQ95713-HQ95717

ABSTRACT

The challenges associated with the retrieval and authentication of ancient DNA (aDNA) evidence are principally due to post-mortem damage which makes ancient samples particularly prone to contamination from "modern" DNA sources. The necessity for authentication of results has led many aDNA researchers to adopt methods considered to be "gold standards" in the field, including cloning aDNA amplicons as opposed to directly sequencing them. However, no standardized protocol has emerged regarding the necessary number of clones to sequence, how a consensus sequence is most appropriately derived, or how results should be reported in the literature. In addition, there has been no systematic demonstration of the degree to which direct sequences are affected by damage or whether direct sequencing would provide disparate results from a consensus of clones.

To address this issue, a comparative study was designed to examine both cloned and direct sequences amplified from ~3,500 year-old ancient northern fur seal DNA extracts. Majority rules and the Consensus Confidence Program was used to generate consensus sequences for each individual from the cloned sequences, which exhibited damage at 31 of 139 base pairs across all clones. In no instance did the consensus of clones differ from the direct sequence. This study demonstrates that, when appropriate, cloning need not be the default method, but instead, should be used as a measure of authentication on a case-by-case basis, especially when this practice adds time and cost to studies where it may be superfluous.

INTRODUCTION

The ability to study DNA from organisms that have been long dead [i.e. ancient DNA (aDNA)], has led to numerous insights into the evolutionary history of humans, animals, plants, and even microorganisms (Green et al., 2010; Hebsgaard et al., 2009; Kemp et al., 2007; Krings et al., 1997; Lambert et al., 2002; Lindqvist et al., 2010; Pääbo et al., 2004; Poinar et al., 2001; Shapiro et al., 2004; Speller et al., 2010; Willerslev et al., 2007; Willerslev and Cooper, 2005; Willerslev et al., 2003; Zeder et al., 2006). The strength of aDNA evidence is affected, however, by its challenging retrieval and authentication, principally as a result of postmortem damage. Degradation by nucleases, oxidation, deamination, depurination, and background radiation lead to destabilization and breaks in DNA strands (Hofreiter et al., 2001) leaving aDNA template molecules typically short in length with chemically modified (i.e. "damaged") nucleotide positions (Gilbert et al., 2006a; Pääbo et al., 1988). Consequently, aDNA studies are prone to contamination from "modern" DNA sources that can completely out-compete endogenous DNA in polymerase chain reaction (PCR) amplification (Kemp and Smith, 2005). These problems are not unique to the aDNA field, but

are also encountered in forensic research where degraded remains and sample mixtures are common (Alonso et al., 2004; Capelli et al., 2003).

Troubled by the overwhelming lack of standards followed by aDNA practitioners that presented at the 5th International Ancient DNA Conference in 2000, Cooper and Poinar (2000), published a very timely opinion piece in *Science* that outlined a list of criteria that should be followed in order to authenticate aDNA evidence for publication (Cooper and Poinar, 2000). The recommendations of Cooper and Poinar (2000) have had a profound impact on the field both positive and, and in some cases, negative. For example, reviewers have rejected manuscripts written by authors that did not follow each and every recommendation of Cooper and Poinar (2000), referring to them as "classical stringent standards" (Kemp and Smith, 2010)This is particularly troubling given the understanding that following despite the fact that subsequent research clearly showed that the recommendations of Cooper and Poinar (2000) alone can not authenticate aDNA evidence (Gilbert et al., 2005; Kemp and Smith, 2010). Additionally, some of their criteria such as amino acid racemization (AAR) have been discounted as a predictor of DNA preservation (Collins et al., 2009; Fernandez et al., 2009), while in contrast, critical decontamination methodologies (e.g. Kemp and Smith, 2005) were never "required". Unfortunately, one of the most critically important points made by Cooper and Poinar (2000), that data produced need to make sense, rarely generates much attention.

This study focuses on the fifth recommendation of Cooper and Poinar (2000), which states "Direct PCR sequences must be verified by cloning amplified products to determine the ratio of endogenous to exogenous sequences, damage-induced errors, and to detect the presence of numts. Overlapping fragments are desirable to confirm that sequence variation is authentic and not the product of errors introduced when PCR amplification starts from a small number of damaged templates". Since publication of Cooper and Poinar's (2000) critique, cloning has become a common practice, yet no standardization has emerged regarding the number of clones required to produce an appropriate consensus, or how to evaluate the validity of the clones that are generated. In addition, there has been no systematic demonstration of the degree to which direct sequences are affected by damage or whether direct sequencing would provide disparate results from a consensus of clones. To address these issues, aDNA was extracted from the remains of five ~3,500 year old northern fur seals (*Callorhinus ursinus*). Results from direct sequencing and cloning of a portion of the mitochondrial cytochrome B gene were compared following a simple majority rules approach. Furthermore, we evaluated the usefulness of the Consensus Confidence Program (CCP) (Bower et al., 2005) in deriving consensus sequences.

Background: Variability and Inconsistency in the Cloning of aDNA

To illustrate the variability of cloning methodologies, Table 1 summarizes the cloning practices of twenty-nine aDNA studies published in various journals over a sixteen-year period (1994-2010). The data indicate tremendous inter-study variability, with researchers reporting as few as two clones to over 100 per amplification. Some researchers chose to clone only a subset of samples from a given archaeological site to evaluate sequencing fidelity (e.g. Kuhn et al., 2010), which suggests that they believe that taphonomic processes are uniform across a site. This is in stark contrast to the notion that sample specific qualities, such as the copy number of target DNA, should dictate the need to practice cloning, namely the preserved copy number of target DNA (Handt et al., 1996; Pääbo et al., 2004).

Also troubling, are studies that report the number of clones sequenced yet do not publish the results (see for example Endicott et al., 2006; Larson et al., 2007; Thomsen et al., 2009) or provide readable sequence data (Kuch et al., 2007). As a result, it is impossible to evaluate the strength of the data generated, despite the fact that these studies followed the cloning recommendation. This suggests that some reviewers are not evaluating the cloning data itself, but are satisfied merely with the fact that the technique was used during the experimental process. It also means that authors need to be more responsible in clearly reporting their data.

Another major problem with current cloning practices relates to how consensus sequences emerge from the cloned sequence data. Methods for determining consensus sequences are highly variable and lacking standardization. Most studies listed in Table 1 took a majority rules approach to building consensus sequences. This supposition suggests that minority sequences, based solely on their minority status within a pool of clones, represent contaminating and/or chemically modified (i.e. damaged) template molecules. Alternately, in an investigation studying DNA extracted from hominid specimens from Southern Siberia, Krause and colleagues (2007) used the minority status (2 of 104 clones) of Neanderthal-like mitochondrial DNA (mtDNA) sequences to initially support identification as non-human. While Krause and colleagues (2007) rightly used additional means to authenticate their species identification, this serves as a reminder that the use and interpretation of cloning, and its results, is variable and that the most important criterion is that the data make sense.

Dealing with highly damaged DNA also raises the question of whether a cloning consensus can and should be combined from two separate extracts. When reactions start from a separate pool of template molecules extracted on different occasions, it is preferable to generate a consensus from the extracts separately and use each as independent confirmation of the other. When low copy number and damage render this strategy impossible, another extract attempt should be made to confirm the piecemeal consensus sequence. Reporting the ambiguities is an option if, after several attempts at confirmation, a consensus cannot be generated (Handt et al., 1996). Ultimately, the act of cloning itself does not make

the data generated any more authentic and the necessity of the technique and the validity of consensus sequences should be closely monitored on a case-by-case basis.

Despite methodological inconsistencies in the field , a glimmer of clarity was provided by Bower and colleagues (2005). These researchers created a freeware program called the “Consensus Confidence Program” which produces a consensus by calculating the percent probability that statistically each nucleotide occurs most frequently, at an individual position, with a confidence level between 70% and 95%. The program requires a minimum input of 12 clones to generate a consensus sequence. While this program is a tool that offers the means to standardize and produce statistically significant consensus results, it is important to highlight that it cannot “verify whether the consensus sequence is authentic” (pg. 2550).

Regardless of very strong encouragement for the use of cloning by aDNA researchers, there has been no systematic demonstration that directly sequenced PCR products would represent anything but the majority rules consensus of a number of clones (Kemp and Smith, 2010). The cloning recommendation of Cooper and Poinar (2000) was adopted as a mandatory default technique by those in the aDNA field without critical evaluation. From the studies described in Table 1, one finds that in only five of the 29 studies did the researchers even compare cloned sequences to direct sequences. In none of these studies did the majority rules consensus sequence differ from the direct sequence.

An original goal of this study was to use published data to compare direct sequences with the consensus of a minimum of 12 clones as determined by the CCP (Bower et al., 2005). This goal was unachievable as none of the five studies sequenced more than ten clones. Nevertheless, the data in the reviewed literature (Table 1) suggest that cloning aDNA amplicons is not necessary in all cases, especially when this practice adds time and cost to studies where it may be superfluous.

Recommendations for maintaining authenticity in aDNA studies are always appreciated, but it may not necessarily be true that cloning is the only way to generate accurate sequence results. Rather, directly sequencing amplicons from independent amplifications and extractions may be sufficient. The goal of this study is to begin the systematic determination of whether a difference, if any, exists between cloning and direct sequencing in order to generate an aDNA consensus sequence. Working specifically with non-human, non-domesticate animal samples decreases the probability that contamination has contributed to these results (Kemp and Smith, 2005; Leonard et al., 2007).

MATERIALS AND METHODS

Between 0.40 and 0.77g of bone was removed from distal end of five northern fur seal (*Callorhinus ursinus*) rib bones (samples are designated 809005, 809007, 809016, 809023 and 809032) using a new dremmel blade for each sample. These samples were excavated from the Amaknak Bridge Site in Unalaska, AK and date to approximately 3,500 years before present (YBP) (Crockford et al., 2004). All DNA extractions and PCR set-up were conducted in the Kemp Ancient DNA Lab at Washington State University. The samples were submerged in 6% w/v sodium hypochlorite for 15 min and rinsed twice with DNA free H_2O to remove surface contamination (Kemp and Smith, 2005). DNA was extracted following Kemp and colleagues (2007) except that the original volume of sample 809023 in EDTA was split in half before the phenol/chloroform step. A 181 base pair (bp) portion of the cytochrome B gene spanning nucleotide positions (nps) 14185-14365 [relative to a complete mtDNA genome, NC_008415 (Arnason et al., 2006)] was PCR amplified with primers: CytB-F CCAACATTCGAAAAGTTCATCC and CytB-R GCTGTGGTGGTGTCTGAGGT (Moss et al., 2006) for quantification by Real Time PCR and for use in direct sequencing and cloning.

Quantification PCRs were performed on sample extracts in an Applied Biosystems 7300 Real Time PCR System using a MAR-labeled probe: 5'-CATTAACAGCTCGCTC-3' (Allelogic). Each 25 µL reaction contained 0.24 mM dNTPs, 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 0.24 µM probe, 0.5 µM ROX reference dye, 0.75 U of Platinum *Taq* polymerase (Invitrogen™), and 5.0 µL of extract at full concentration, 20%, and 10% to determine levels of inhibition and ensure accuracy of copy numbers. Cycling was performed with an initial 10 minute hold at 95°C followed by 50 cycles of 15 seconds at 95°C and 60 seconds at 55°C. A minimum of 4 negative template controls were included on each 96-well plate to monitor contamination in reagents and ROX-labeled passive reference dye was included to correct for variation in well-to-well background fluorescence. Amplification curves were analyzed with the automatic baseline feature of the 7300 System SDS software (Applied Biosystems) with an empirically determined threshold of 0.05. Calibration curves were generated from a freshly prepared serial dilution series of standard DNA amplified from modern northern fur seal whole genomic DNA extract. Slopes of the calibration curves were used to calculate assay efficiencies (%PCR efficiency = $(10^{(-1/slope)} - 1) \times 100$) and all were required to meet an efficiency > 87% with $R^2 > 0.996$ for data inclusion. Analyzed data were exported from the 7300 SDS software into a CSV file (comma delimited) for secondary analysis and formatting in Microsoft® Excel 2007.

Amplifications for direct sequencing and cloning contained 0.32 mM dNTPs, 1X PCR Buffer, 1.5 mM, 1.5 mM $MgCl_2$, 0.24 µM of each primer, 0.3 U of Platinum *Taq* polymerase (Invitrogen™), and 3.0 µL of DNA template in 30 µL reactions. These reactions were subjected to 60 cycles of PCR as follows: 3 min denaturing at 94°C, followed by 15 second holds at 94°C, 55°C, and at 72°C, with a final 3 min extension period at 72°C. Negative control amplifications were carried out to

detect potential contamination. Two independent PCR amplifications from each of the five extracts were submitted for direct sequencing. One microliter from each amplification was then cloned using a TOPO® TA cloning kit and TOP10 competent cells (Invitrogen™) following manufacturer's instructions with the exception that reactions were scaled to one quarter. A minimum of 16 white colonies were selected from each sample transformation and underwent colony PCR using the CytB primers for the first transformation and M13 primers for the second transformation. Colony PCRs were the same as above except they were prepared for a 15 µL reaction with 1.5 µL of DNA template, and the M13 primers were cycled with an annealing temperature of 58°C. Control plates and transformation of PCR negatives were used to ensure cell competency and PCR amplifications free of contamination. Clones containing the transformed vector were then sequenced at a minimum of 13 clones per sample. All amplicons were prepared for sequencing and purified using a Multiscreen $PCR_{\mu 96}$ filter plate (Millipore). Amplicons were brought to a volume of 100 µL, using dH_20, before transfer to the filter plate. After vacuuming, 25 µL of dH_20 was added to each well, followed by 30 minutes of shaking at 350 rpms. Direct sequencing was performed in both directions at the DNA Analysis Facility at Yale University. Sequences were aligned to a complete northern fur seal mtDNA reference sequence (Genbank accession number NC_008415 from Arnason et al., 2006) using Sequencher® 4.8.

As mtDNA does not undergo recombination, the majority rules consensus sequence was determined to be the haplotype present in greater than 50% of the clones. Cloned sequences from the five samples were analyzed by the CCP (Bower et al., 2005) to determine percent confidence and any variation(s) from the majority rule consensus sequence. The direct sequence was then compared to each consensus sequence.

As an additional control, a third PCR amplification was directly sequenced as described above, but not cloned, for comparison to the first two direct sequences and to that of the consensus sequences determined from the sequenced clones from these PCRs.

RESULTS

Deviations among the clones from the consensus were observed at a total of 31 sites within 150 cloned fragments across the five samples (Table 2). Single base polymorphisms that appear as "transitions" in the clones, when compared to the direct sequence, were recorded as damage. The majority of the damaged sites were C>T, which is indicative of deamination (Hofreiter et al., 2001). Sites with double peaked base pairs in the cloned sequences (designated as N) must represent errors that arose during colony growth or subsequent PCR (see Figure 1 for an example).

None of the five samples showed any difference between the direct sequence, the majority rules consensus, and the consensus as determined by the CCP (Table 2). The sole exception to this finding is the second transformation of sample 809023, where a majority rules consensus could not be determined because the most common haplotype was present in only 5 of 12 clones (41.7%). Similiarly, a 95% confidence consensus from the CCP could not be determined due to the high number of unique sequences among the clones. The direct sequence for this transformation does however, accurately reflect the mix of cloned sequences. That is, the competition of peak intensities at the N sites in the direct sequence correlate with positions in the clones that reveal a substantial mix of adenines and guanines. For example, at site 14281, 10 of out of 15 clones show an A instead of a G (Table 2) and the chromatogram shows competing A and G peaks (see Figure 1). In this case, the third independent PCR amplification was consistent with the first PCR amplification and first transformation (Table 2).

The quantification of samples shows a diverse range of average copies of mtDNA per microliter from 35 (SD 4) to 1737 (SD 333) (Table 2).

DISCUSSION

While we have chosen here to work with non-human, non domestic animal samples, and this is the first study to demonstrate that directly sequencing aDNA can provide the same data as taking a consensus of clones (as assessed by a majority rules approach, the CCP, or both), it is prudent to mention that our results cannot be extrapolated across all studies. For instance there are cases in aDNA research where cloning is an absolute necessity. Without relying on the capacity of next generation sequencing, cloning would be, for example, the only means of reconstructing ancient diets from DNA preserved in coprolites (Poinar et al., 1998; Poinar et al., 2001), or studying a mixture of DNA extracted from soil (Hebsgaard et al., 2009; Willerslev et al., 2003) or ice samples (Willerslev et al., 2007). The reason that cloning is essential in these cases is that their goal is to observe as many unique molecules as permitted, not to reach a consensus sequence from a pool of clones. In contrast, the focus of this initial study was deriving a consensus sequence from endogenous molecules from single individuals.

Cloning would also be necessary if the goal of a study is to derive an aDNA sequence from a heavily contaminated sample that cannot be decontaminated prior to DNA extraction. For example, there has been no demonstration that human coprolites can be efficiently decontaminated, which is why cloning was necessary to conclude that the coprolites excavated from Paisley Caves were produced by the occupants of the caves (Gilbert et al., 2008). However, this conclusion was not drawn from taking a consensus of a pool of clones, rather it relied on knowledge about the mtDNA mutations exhibited by the first Americans, relative to those exhibited by non-Native Americans. In contrast, if a sample can

be sufficiently decontaminated [e.g. bone or tooth samples (Kemp and Smith, 2005; but see Malmstrom et al., 2007; Salamon et al., 2005)] cloning may be less necessary in deriving an individual sequence. While the experiment was not conducted, it would have been very interesting if Krause and colleagues (2007) had decontaminated a piece of Neanderthal bone, and extracted and analyzed this in parallel with the samples that they did not decontaminate. Then the results from the decontaminated bone could be compared against the 98% contamination they observed in the clones (102 of 104) of their experiment. Again, the experiments in this study were not conducted to address this issue; therefore, the results are not directly applicable to either of these scenarios. Future studies that explore the relationship between decontamination and cloning are necessary.

Cloning remains an appropriate and reliable method for obtaining aDNA sequences, given that this practice has the potential for showing the composition of a mixed PCR reaction (whether the heterogeneity of molecules arose from damage or contamination). However, as shown here in the second amplification of sample 809023, direct sequencing also permits one to see that the authenticity of a sequence is compromised by having started from a highly heterogeneous pool of molecules (i.e. when double peaks are present in the chromatograms). This amplification of the sample shows that even with competing damage, both approaches will yield the same result, and would require an additional amplification to reach a consensus for the sequence. Given the results presented,we argue that cloning should not serve as the default first step method for obtaining consensus sequences from aDNA samples, as has become commonplace in the field. This is especially true considering that direct sequencing is more time and cost efficient and, thus, could hasten discovery and publication.

While there is a general “rule” in the aDNA field that one should be suspicious of sequences initiated from a pool of less than 1000 template molecules (Pääbo et al., 2004), our study has shown that even very low copy number samples [35 copies/ µL (SD 4)] can provide reliable direct sequences. This “1000 molecule rule” originated from a study conducted by Handt and colleagues (1996), who actually stated “A minimum of 100-1,000 molecules per amplification” (pg. 375) may be needed to get around the problems of sporadic contamination and/or damaged template molecules. As this cut off was determined with much cruder methods than are available today, we suggest that the relationship between the number of starting template molecules in a PCR and the reliability of the resulting sequence (whether produced directly or from a consensus of clones) needs re-evaluation. We anticipate that the repeatability of data will be more crucial to determining authenticity than starting template molecule copy numbers (Gilbert et al., 2009), an expectation which is supported by the reliability of the sequence derived from our lowest copy number sample (809023).

Ancient DNA research is positioned to continue to provide answers to questions of the past but, as most practitioners in the field recognize, collection and authentication of results will always be a challenge. With all the problems and circumstances associated with aDNA, researchers must be proactive in minimizing inaccurate results that can lead to dubious claims. While the recommendations of Cooper and Poinar (2000), or any other list of recommendations, were created as well intentioned advice for ensuring accurate results, they should not act as a simple checklist for researchers to follow and reviewers to note (Gilbert et al., 2005). We do not outright reject these recommendations because in practice they are aimed at reducing contamination and strengthening evidence that the molecules are, in fact, ancient. However, following the rationale outlined by Gilbert and colleagues (2005) and Kemp and Smith (2010), and supported by the data presented here, we disagree that protocols in the aDNA field should be dictated by a methods checklist. We recommend that researchers be as explicit as possible in describing their methods as well as their rationale for using them. It is appropriate for researchers to state their reason for cloning, other than just to satisfy the requirements suggested by Cooper and Poinar (2000) (for example was done by Handt et al., 1996; Handt et al., 1994). This allows the reader to better understand the characteristics of the sample and the critical analysis that contributed to making methodological choices. For example, if the research question relies on the knowledge that a PCR reaction began from a heterogenous pool of molecules, cloning would be an appropriate method to confirm this. However, as demonstrated here, generating a sequence from an ancient sample does not require cloning and, as such, the method need not serve as the default approach.

Ancient DNA data should be evaluated according to the specific methods used to generate them, paying particular attention to the degree to which the data make sense. A cognitive approach to aDNA is necessary for assessing the reliability of results. Each study has specific problems and criteria that need to be considered in order to advocate reliable data. The “Key questions to ask about ancient DNA” (pg 543) as suggested by Gilbert and colleagues (2005) throws out the idea of a requirements checklist and instead proposes that readers, reviewers, and authors alike analyze whether or not the results make sense within the context of the study. Similarly, the results presented here underscore the point that rather than employing a methods checklist, reviewers need to more critically appraise the data that are presented in a study in order to judge the quality of research.

ACKNOWLEDGMENTS

Thank you to Dr. Susan J. Crockford for turning our attention to the usefulness of ancient northern fur seals in these experiments. Thanks to Dr. Melissa Goodman-Elgar for allowing us to use her lab space to cut the ribs. Special thanks to the Seattle Aquarium for providing modern northern fur seal samples to use for standards and positive controls. Thank you to Dr. Tom Gilbert for his critical evaluation of our manuscript as well as the very helpful comments and critiques from two anonymous reviewers.

WORKS CITED

Alonso A, Martin P, Albarran C, Garcia P, Garcia O, de Simon LF, Garcia-Hirschfeld J, Sancho M, de la Rua C, and Fernandez-Piqueras J (2004) Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. Forensic Science International 139:141-149.

Arnason U, Gullberg A, Janke A, Kullberg M, Lehman N, Petrov E, and Väinölä R (2006) Pinniped phylogeny and a new hypothesis for their origin and dispersal. Molecular phylogenetics and evolution 41:345-354.

Binladen J, Wiuf C, Gilbert MT, Bunce M, Barnett R, Larson G, Greenwood AD, Haile J, Ho SY, Hansen AJ, and Willerslev E (2006) Assessing the fidelity of ancient DNA sequences amplified from nuclear genes. Genetics 172:733-41.

Bouwman AS, and Brown TA (2005) The limits of biomolecular palaeopathology: ancient DNA cannot be used to study venereal syphilis. Journal of Archaeological Science 32:703-713.

Bower MA, Spencer M, Matsumura S, Nisbet RER, and Howe CJ (2005) How many clones need to be sequenced from a single forensic or ancient DNA sample in order to determine a reliable consensus sequence? Nucleic Acids Research 33:2549-2556.

Capelli C, Tschentscher F, and Pascali VL (2003) "Ancient" protocols for the crime scene? Similarities and differences between forensic genetics and ancient DNA analysis. Forensic Science International 131:59-64.

Caramelli D, Lalueza-Fox C, Vernesi C, Lari M, Casoli A, Mallegni F, Chiarelli B, Dupanloup I, Bertranpetit J, Barbujani G, and Bertorelle G (2003) Evidence for a genetic discontinuity between Neandertals and 24,000-year-old anatomically modern Europeans. Proc Natl Acad Sci U S A 100:6593-7.

Collins MJ, Penkman KE, Rohland N, Shapiro B, Dobberstein RC, Ritz-Timme S, and Hofreiter M (2009) Is amino acid racemization a useful tool for screening for ancient DNA in bone? Proc Biol Sci 276:2971-7.

Cooper A, and Poinar HN (2000) Ancient DNA: do it right or not at all. Science 289:1139.

Crockford SJ, Fredrick G, Wigen R, and McKechnie I (2004) Final Report on the analysis of the vertebrate fauna from Amaknak Bridge, Unalaska, AK, UNL050: Project # MGS-STP-BR-0310(S)/52930 to R. Knecht and R. Davis. Report on file, Alaska Department of Transportation, Anchorage.

Endicott P, Metspalu M, Stringer C, Macaulay V, Cooper A, and Sanchez JJ (2006) Multiplexed SNP typing of ancient DNA clarifies the origin of Andaman mtDNA haplogroups amongst South Asian tribal populations. PLoS One 1:e81.

Fernandez E, Ortiz JE, Perez-Perez A, Prats E, Turbon D, Torres T, and Arroyo-Pardo E (2009) Aspartic acid racemization variability in ancient human remains: implications in the prediction of ancient DNA recovery. Journal of Archaeological Science 36:965-972.

Gilbert M, Bandelt H, Hofreiter M, and Barnes I (2005) Assessing ancient DNA studies. Trends in Ecology & Evolution 20:541-544.
Gilbert MT, Binladen J, Miller W, Wiuf C, Willerslev E, Poinar H, Carlson JE, Leebens-Mack JH, and Schuster SC (2006a) Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis. Nucleic Acids Res 35:1-10.
Gilbert MT, Menez L, Janaway RC, Tobin DJ, Cooper A, and Wilson AS (2006b) Resistance of degraded hair shafts to contaminant DNA. Forensic Sci Int 156:208-12.
Gilbert MT, Wilson AS, Bunce M, Hansen AJ, Willerslev E, Shapiro B, Higham TF, Richards MP, O'Connell TC, Tobin DJ, Janaway RC, and Cooper A (2004) Ancient mitochondrial DNA from hair. Curr Biol 14:R463-4.
Gilbert MTP, Jenkins DL, Gotherstrom A, Naveran N, Sanchez JJ, Hofreiter M, Thomsen PF, Binladen J, Higham TFG, Yohe RM, II, Parr R, Cummings LS, and Willerslev E (2008) DNA from pre-Clovis human coprolites in Oregon, North America. Science 320:786-9.
Gilbert MTP, Jenkins DL, Higham TFG, Rasmussen M, Malmstrom H, Svensson EM, Sanchez JJ, Cummings LS, Yohe RM, II, Hofreiter M, Gotherstrom A, and Willerslev E (2009) Response to Comment by Poinar et al. on "DNA from Pre-Clovis Human Coprolites in Oregon, North America". Science 325:148-b.
Green RE, Krause J, Briggs AW, Maricic T, Stenzel U, Kircher M, Patterson N, Li H, Zhai W, Fritz MH, Hansen NF, Durand EY, Malaspinas AS, Jensen JD, Marques-Bonet T, Alkan C, Prufer K, Meyer M, Burbano HA, Good JM, Schultz R, Aximu-Petri A, Butthof A, Hober B, Hoffner B, Siegemund M, Weihmann A, Nusbaum C, Lander ES, Russ C, Novod N, Affourtit J, Egholm M, Verna C, Rudan P, Brajkovic D, Kucan Z, Gusic I, Doronichev VB, Golovanova LV, Lalueza-Fox C, de la Rasilla M, Fortea J, Rosas A, Schmitz RW, Johnson PL, Eichler EE, Falush D, Birney E, Mullikin JC, Slatkin M, Nielsen R, Kelso J, Lachmann M, Reich D, and Paabo S (2010) A draft sequence of the Neandertal genome. Science 328:710-22.
Green RE, Malaspinas AS, Krause J, Briggs AW, Johnson PL, Uhler C, Meyer M, Good JM, Maricic T, Stenzel U, Prufer K, Siebauer M, Burbano HA, Ronan M, Rothberg JM, Egholm M, Rudan P, Brajkovic D, Kucan Z, Gusic I, Wikstrom M, Laakkonen L, Kelso J, Slatkin M, and Paabo S (2008) A complete Neandertal mitochondrial genome sequence determined by high-throughput sequencing. Cell 134:416-26.
Haak W, Forster P, Bramanti B, Matsumura S, Brandt G, Tanzer M, Villems R, Renfrew C, Gronenborn D, Alt KW, and Burger J (2005) Ancient DNA from the first European farmers in 7500-year-old Neolithic sites. Science 310:1016-8.
Handt O, Krings M, Ward RH, and Paabo S (1996) The retrieval of ancient human DNA sequences. American Journal of Human Genetics 59:368-376.

Handt O, Richards M, Trommsdorff M, Kilger C, Simanainen J, Georgiev O, Bauer K, Stone A, Hedges R, Schaffner W, and et al. (1994) Molecular genetic analyses of the Tyrolean Ice Man. Science 264:1775-8.

Hebsgaard MB, Gilbert MTP, Arneborg J, Heyn P, Allentoft ME, Bunce M, Munch K, Schweger C, and Willerslev E (2009) "The Farm Beneath the Sand"- and archaeological case study on ancient "dirt" DNA. Antiquity 83:430-444.

Helgason A, Lalueza-Fox C, Ghosh S, Sigurethardottir S, Sampietro ML, Gigli E, Baker A, Bertranpetit J, Arnadottir L, Thornorsteinsdottir U, and Stefansson K (2009) Sequences from first settlers reveal rapid evolution in Icelandic mtDNA pool. PLoS Genet 5:e1000343.

Hofreiter M, Capelli C, Krings M, Waits L, Conard N, Munzel S, Rabeder G, Nagel D, Paunovic M, Jambresic G, Meyer S, Weiss G, and Paabo S (2002) Ancient DNA analyses reveal high mitochondrial DNA sequence diversity and parallel morphological evolution of late pleistocene cave bears. Mol Biol Evol 19:1244-50.

Hofreiter M, Jaenicke V, Serre D, von Haeseler A, and Pääbo S (2001) DNA sequence from mulitple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. Nucleic Acids Research 29:4793-4799.

Karanth KP, Delefosse T, Rakotosamimanana B, Parsons TJ, and Yoder AD (2005) Ancient DNA from giant extinct lemurs confirms single origin of Malagasy primates. Proc Natl Acad Sci U S A 102:5090-5.

Kemp B, Malhi R, McDonough J, Bolnick D, Eshleman J, Rickards O, Martinez-Labarga C, Johnson J, Lorenz J, and Dixon E (2007) Genetic analysis of early Holocene skeletal remains from Alaska and its implications for the settlement of the Americas. American Journal of Physical Anthropology 132:605.

Kemp BM, and Smith DG (2005) Use of Bleach to Eliminate Contaminating DNA from the Surfaces of Bones and Teeth. Forensic Science International 154:53-61.

Kemp BM, and Smith DG (2010) Ancient DNA Methodology: "Thoughts from Brian M. Kemp and David Glenn Smith on "Mitochondrial DNA of Protohistoric Remains of an Arikara Population from South Dakota". Human Biology 82:227-238.

Kim JH, Oh JH, Song JH, Jeon JT, Han SH, Jung YH, and Oh MY (2005) Molecular genetic analysis of ancient cattle bones excavated from archaeological sites in Jeju, Korea. Mol Cells 20:325-30.

Krause J, Orlando L, Serre D, Viola B, Prufer K, Richards MP, Hublin JJ, Hanni C, Derevianko AP, and Paabo S (2007) Neanderthals in central Asia and Siberia. Nature 449:902-4.

Krings M, Geisert H, Schmitz RW, Krainitzki H, and Paabo S (1999) DNA sequence of the mitochondrial hypervariable region II from the neandertal type specimen. Proc Natl Acad Sci U S A 96:5581-5.

Krings M, Stone A, Schmitz RW, Krainitzki H, Stoneking M, and Paabo S (1997) Neandertal DNA sequences and the origin of modern humans. Cell 90:19-30.

Kuch M, Grocke DR, Knyf MC, Gilbert MT, Younghusband B, Young T, Marshall I, Willerslev E, Stoneking M, and Poinar H (2007) A preliminary analysis of the DNA and diet of the extinct Beothuk: A systematic approach to ancient human DNA. Am J Phys Anthropol 132:594-604.

Kuhn TS, McFarlane KA, Groves P, Mooers AO, and Shapiro B (2010) Modern and ancient DNA reveal recent partial replacement of caribou in the southwest Yukon. Mol Ecol 19:1312-23.

Lambert DM, Ritchie PA, Millar CD, Holland B, Drummond AJ, and Baroni C (2002) Rates of evolution in ancient DNA from Adelie penguins. Science (Washington D C) 295:2270-2273.

Lari M, Rizzi E, Milani L, Corti G, Balsamo C, Vai S, Catalano G, Pilli E, Longo L, Condemi S, Giunti P, Hanni C, De Bellis G, Orlando L, Barbujani G, and Caramelli D (2010) The microcephalin ancestral allele in a Neanderthal individual. PLoS One 5:e10648.

Larson G, Albarella U, Dobney K, Rowley-Conwy P, Schibler J, Tresset A, Vigne JD, Edwards CJ, Schlumbaum A, Dinu A, Balacsescu A, Dolman G, Tagliacozzo A, Manaseryan N, Miracle P, Van Wijngaarden-Bakker L, Masseti M, Bradley DG, and Cooper A (2007) Ancient DNA, pig domestication, and the spread of the Neolithic into Europe. Proc Natl Acad Sci U S A 104:15276-81.

Leonard JA, Shanks O, Hofreiter M, Kreuz E, Hodges L, Ream W, Wayne RK, and Fleischer RC (2007) Animal DNA in PCR reagents plagues ancient DNA research. Journal of Archaeological Science 34:1361-1366.

Lindqvist C, Schuster SC, Sun Y, Talbot SL, Qi J, Ratan A, Tomsho LP, Kasson L, Zeyl E, Aars J, Miller W, Ingolfsson O, Bachmann L, and Wiig O (2010) Complete mitochondrial genome of a Pleistocene jawbone unveils the origin of polar bear. Proc Natl Acad Sci U S A 107:5053-7.

Loreille O, Orlando L, Patou-Mathis M, Philippe M, Taberlet P, and Hanni C (2001) Ancient DNA analysis reveals divergence of the cave bear, Ursus spelaeus, and brown bear, Ursus arctos, lineages. Curr Biol 11:200-3.

Malmstrom H, Stora J, Dalen L, Holmlund G, and Gotherstrom A (2005) Extensive human DNA contamination in extracts from ancient dog bones and teeth. Mol Biol Evol 22:2040-7.

Malmstrom H, Svensson EM, Gilbert MT, Willerslev E, Gotherstrom A, and Holmlund G (2007) More on contamination: the use of asymmetric molecular behavior to identify authentic ancient human DNA. Mol Biol Evol 24:998-1004.

Monsalve MV, Stone AC, Lewis CM, Rempel A, Richards M, Straathof D, and Devine DV (2002) Brief communication: molecular analysis of the Kwaday Dan Ts'finchi ancient remains found in a glacier in Canada. Am J Phys Anthropol 119:288-91.

Moss M, Yang D, Newsome S, Speller C, McKechnie I, McMillan A, Losey R, and Koch P (2006) Historical ecology and biogeography of North Pacific pinnipeds: Isotopes and ancient DNA from three archaeological assemblages. The Journal of Island and Coastal Archaeology 1:165-190.

Orlando L, Darlu P, Toussaint M, Bonjean D, Otte M, and Hanni C (2006) Revisiting Neandertal diversity with a 100,000 year old mtDNA sequence. Curr Biol 16:R400-2.
Orlando L, Leonard JA, Thenot A, Laudet V, Guerin C, and Hanni C (2003) Ancient DNA analysis reveals woolly rhino evolutionary relationships. Mol Phylogenet Evol 28:485-99.
Ovchinnikov IV, Gotherstrom A, Romanova GP, Kharitonov VM, Liden K, and Goodwin W (2000) Molecular analysis of Neanderthal DNA from the northern Caucasus. Nature 404:490-3.
Pääbo S, Gifford JA, and Wilson AC (1988) Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Research 16:9775-9788.
Pääbo S, Poinar H, Serre D, Jaenicke-Després V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, and Hofreiter M (2004) Genetic analyses from ancient DNA. Annual Review of Genetics 38:645-679.
Poinar H, Kuch M, McDonald G, Martin P, and Paabo S (2003) Nuclear gene sequences from a late Pleistocene sloth coprolite. Current Biology 12:1150-1152.
Poinar HN, Hofreiter M, Spaulding WG, Martin PS, Stankiewicz BA, Bland H, Evershed RP, Possnert G, and Paabo S (1998) Molecular coproscopy: Dung and diet of the extinct ground sloth Nothrotheriops shastensis. Science (Washington D C) 281:402-406.
Poinar HN, Kuch M, Sobolik KD, Barnes I, Stankiewicz AB, Kuder T, Spaulding WG, Bryant VM, Cooper A, and Paabo S (2001) A molecular analysis of dietary diversity for three archaic Native Americans. Proceedings of the National Academy of Sciences of the United States of America 98:4317-4322.
Salamon M, Tuross N, Arensburg B, and Weiner S (2005) Relatively well preserved DNA is present in the crystal aggregates of fossil bones. Proc Natl Acad Sci U S A 102:13783-13788.
Shapiro B, Drummond AJ, Rambaut A, Wilson MC, Matheus PE, Sher AV, Pybus OG, Gilbert MT, Barnes I, Binladen J, Willerslev E, Hansen AJ, Baryshnikov GF, Burns JA, Davydov S, Driver JC, Froese DG, Harington CR, Keddie G, Kosintsev P, Kunz ML, Martin LD, Stephenson RO, Storer J, Tedford R, Zimov S, and Cooper A (2004) Rise and fall of the Beringian steppe bison. Science 306:1561-5.
Speller CF, Kemp BM, Wyatt SD, Monroe C, Lipe WD, Arndt UM, and Yang DY (2010) Ancient Mitochondrial DNA Analysis Reveals Complexity of Indigenous North American Turkey Domestication. Proceedings of the National Academy of Science USA 107:2807-2812.
Thomsen PF, Elias S, Gilbert MT, Haile J, Munch K, Kuzmina S, Froese DG, Sher A, Holdaway RN, and Willerslev E (2009) Non-destructive sampling of ancient insect DNA. PLoS One 4:e5048.
Willerslev E, Cappellini E, Boomsma W, Nielsen R, Hebsgaard MB, Brand TB, Hofreiter M, Bunce M, Poinar HN, Dahl-Jensen D, Johnsen S, Steffensen JP, Bennike O, Schwenninger JL, Nathan R, Armitage S, de Hoog CJ, Alfimov V, Christl M, Beer J, Muscheler R, Barker J, Sharp M, Penkman

KE, Haile J, Taberlet P, Gilbert MT, Casoli A, Campani E, and Collins MJ (2007) Ancient biomolecules from deep ice cores reveal a forested southern Greenland. Science 317:111-4.
Willerslev E, and Cooper A (2005) Ancient DNA. Proc Biol Sci 272:3-16.
Willerslev E, Hansen AJ, Binladen J, Brand TB, Gilbert MT, Shapiro B, Bunce M, Wiuf C, Gilichinsky DA, and Cooper A (2003) Diverse plant and animal genetic records from Holocene and Pleistocene sediments. Science 300:791-5.
Zeder MA, Emshwiller E, Smith BD, and Bradley DG (2006) Documenting domestication: the intersection of genetics and archaeology. Trends Genet 22:139-55.

Figure 1. Chromatogram for site 14281 on sample 809023

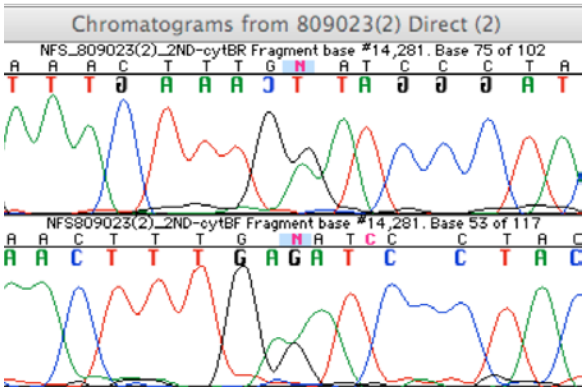


Table 1. Example of studies that utilized cloning in the study if aDNA sorted by year of publication.

		Number of Clones Sequenced											
Study	**Comparison to Direct Sequence?**	**2**	**3**	**4-5**	**6-8**	**9-11**	**12-20**	**21-40**	**41-60**	**61-80**	**81-100**	**101+**	**Species/Samples**
Handt et al (1994)	N						X						Human (Tyrolean Iceman)
Handt et al (1996)	N		X	X	X								Human
Krings et al (1997)	N			X	X	X	X						Neanderthal
Poinar et al (1998)	N				X	X	X						Ground Sloth
Krings et al (1999)	N				X	X	X						Neanderthal
Ovchinnikov et al (2000)	Y		X	X									Neanderthal
Hofreiter et al (2001)	N				X	X							Cave Bear
Loreille et al (2001)	Y		X	X	X								Cave Bear and Brown Bear
Hofreiter et al (2002)	N		X	X	X	X	X						Cave bear
Monsalve et al (2002)	Y			X	X								Human
Caramelli et al (2003)	N			X	X	X							Human
Orlando et al (2003)	N	X	X	X	X								Woolly rhinoceros
Poinar et al (2003)	N				X	X	X						Sloth
Gilbert et al (2004)	N			X	X	X	X						Human
Bouwman and Brown (2005)	N			X									Humans, Syphilis
Haak et al (2005)	N			X	X	X	X						Human
Jae-Hwan et al (2005)	N		X										Cows
Karanth et al (2005)	N		X	X									Lemurs
Malmstrom et al (2005)	N								X				Human, Dog
Salamon et al (2005)	N			X	X								Cat, Penguin, Human
Binladen et al (2006)	Y			X		X							Woolly Rhinoceros, Lion, Pig, Moa
Gilbert et al (2006b)	N				X	X			X	X			Human
Orlando et al (2006)	N				X		X						Neanderthal
Krause et al (2007)	N		X		X		X				X	X	Neanderthal
Kuch et al (2007)*	N			X	X								Human
Green et al (2008)	N											X	Neanderthal
Helgason et al (2009)	N	X	X	X	X	X	X	X	X	X			Human
Kuhn et al (2010)	Y					X							Caribou
Lari et al (2010)	N						X	X					Neanderthal

Categories for number of clone sequences were arbitrarily chosen.
*estimated number of clones from Figure 3 (Kuch et al., 2007)

Table 2. Results from the sequencing and quantification of samples.

Sample	Copy # /µL	S.D.	Amplication #	Direct Sequence	Majority Rules	CCP	Clones
809005	644	9.784	1	14285 A	14285 A	14285 A	13:14285A; **1:14285N**
			2	14285 A	14285 A	14285 A	10:14285A; 1:14285A, **14293T**; 1:14285A, **14322A**
			3	14285 A	NA	NA	Not cloned
809007	1737	332.96	1	Reference	Reference	Reference	14:Reference; **1:14285N, 14289N**; **1:14300N**
			2	Reference	Reference	Reference	14:Reference; **1:14210A, 14227A, 14231A, 14247A, 14316T**; **1:14241T, 14244T, 14245T, 14246T, 14284T**
			3	Reference	NA	NA	Not cloned
809016	91	29.132	1	Reference	Reference	Reference	15:Reference; **1:14223G**
			2	Reference	Reference	Reference	15:Reference; **1:14334A**
			3	Reference	NA	NA	Not cloned
809023	35	3.868	1	Reference	Reference	Reference	10:Reference; **1:14210A, 14281A,14339N**; **1:14227A, 14265A, 14280A, 14304A**: **1:14281N**
			2	**14227N, 14231N, 14265N, 14269N, 14281N**	None	None: Did not meet 95% confidence	**5:14227A, 14231A, 14265A, 14269A, 14281A, 14304A, 14334A; 3:14227A, 14231A; 2:14210A, 14211A, 14265A, 14281A, 14299A, 14232A,14233A; 1:14227A, 14231A,14265A, 14269A, 14281A, 14304A; 1:14210A, 14211A, 14334A; 1:14265A, 14269A,14334A; 1:14280A, 14281A, 14299A, 14304A, 14334A; 1:14265A, 14269A, 14281A, 14304A, 14334A**
			3	Reference	NA	NA	Not cloned
809032	115	42.496	1	14285A	14285A	14285A	11: 14285A; **2:14227A**, 14285A; **1:14250N**, 14285A; **1:142545T**, 14285A; 1:14285A, **14334A**
			2	14285A	14285A	14285A	14:14285A; **1:14227A**, 14285A; **1:Reference**
			3	14285 A	NA	NA	Not cloned

Samples were sequenced from nps 14207 – 14345, relative to a complete mtDNA genome, NC_008415 (Arnason et al., 2006), and polymorphisms listed (not in bold) are relative to that reference sequence. For the clone category, results should be read as follows: the first number refers to the number of clones that have the damage/error, the second number provides the position in the sequence followed by the letter of the base pair that is now seen (e.g. 13:14285A reads as 13 clones with an adenine present at site 14285). Quantification results represent the average copy number over duplicate qPCR reactions and the standard deviation is reported. Note that qPCR results are indicative of the relative DNA level, but should not be taken as exact quantification.

Cloning May Not Be a Necessary Criterion for the Authentication of Ancient DNA Consensus Sequences and Damage Appears to be Randomly Distributed Across the Human Mitochondrial HVRI Region

Misa Winters[1], Cara Monroe[1,2,3], and Brian M. Kemp[1,2]

[1] School of Biological Sciences, Washington State University, Pullman, WA 99164
[2] Department of Anthropology, Washington State University, Pullman, WA 99164
[3] Department of Anthropology, University of California-Santa Barbara, Santa Barbara, CA 93106

*Corresponding Author:

Brian M. Kemp
Department of Anthropology
Washington State University
Pullman, WA 99164
Office: 509-335-7403
Fax: 509-335-3999
bmkemp@wsu.edu

Keywords: cloning, ancient DNA, authentication, post mortem damage

ABSTRACT

Sequencing clones from PCR amplifications has become a method preferred over directly sequencing PCR products by many ancient DNA (aDNA) researchers. Yet, recently it has shown that directly sequencing ancient mammalian mitochondrial DNA (mtDNA) will not yield a disparate consensus sequence compared with cloning. A similar study has yet to be conducted on human samples. In this study cloned and directly sequenced mitochondrial hypervariable region I (HVRI) amplicons from ~220 to 6000 year old ancient human remains were compared. In no instance did the consensus of clones offer any added confirmation to the endogenous DNA sequence than did the direct sequence. From these same data, a relative rate of nucleotide damage was estimated across 255 cloned fragments and a comparison of expected to observed counts of substitutions was analyzed using a goodness of fit test. Post mortem nucleotide damage across HVRI was shown not to deviate statistically from a random distribution ($G = 6.4992_{df=5}$, $P = 0.2606$). Moreover, there was not a strong relationship between damage hotspots and mutational hotspots, as previously argued for human mitochondrial DNA.

INTRODUCTION

Cloning vs. Direct Sequencing

The ability to study DNA from organisms that have long since died, as well as other biological material preserved in the archaeological and/or paleontological records has opened a genetic window to the past. Researchers are becoming less limited in understanding evolutionary history since it is not necessary to rely solely on the fossil record and morphological evidence of past species, but they can instead use ancient DNA (aDNA) to explore genetic change across both time and space.

However, use of these materials is not without risk or stipulation as both the success in retrieval and behavior of aDNA is highly variable. This can make aDNA results difficult, or sometimes impossible, to authenticate. Due to the typically degraded and damaged state of aDNA molecules, researchers struggle to produce authentic results. Some argue that following a recommended set of standards (e.g. Cooper and Poinar, 2000) helps to ensure that end results are free of contamination and discrepancy, however, there is no guarantee that following a list of guidelines ensures authentication of the data (Gilbert et al., 2005b; Kemp and Smith, 2010). Regardless, some reviewers will reject manuscripts if the authors have not subjected their analysis to this specific standardized set of recommendations, even if the researchers have used other, but (equally) justifiable and acceptable means to authenticate their results, and the data make phylogenetic sense (Kemp and Smith, 2010). It is therefore judicious to test some of these recommendations and provide evidence of their effectiveness, as well as determine if alternative methods of authentication that save both time and resources are viable.

This study focuses on the fifth recommendation of standards as described by Cooper and Poinar (2000), which states that aDNA sequences must be verified through the process of cloning amplified products and sequencing the clones with the goal of identifying damage induced errors and potential mixture of endogenous and exogenous molecules. Cloning is referred to here as the insertion of amplicons into bacterial plasmids which are then further amplified in a bacterial host during colony growth

(typically using commercial kits). Target DNA is extracted from the colonies derived from these transformations, and further amplified and sequenced. In most cases, a consensus is determined from multiple clone sequences using a majority rules approach. This is suggested to be a method to rule out error (i.e., damage) and contamination. However, the use of cloning is highly variable and has been shown to be unnecessary in some cases (Winters et al., 2011). Cloning is a costly and time consuming method, often the most variable in methodological practice, and commonly used despite any conclusive experiments that describe its effectiveness. Furthermore, cloning has become a “gold standard” such that many researchers only use it to fulfill the authentication checklist (Gilbert et al., 2005b) and not because it adds strength to their data.

While cloning has become a common practice in the aDNA field, empirical research has demonstrated that deriving a consensus sequence via direct Sanger sequencing will produce the same result as cloning when analyzing aDNA sequences of non-domesticated animals (Winters et al., 2011). Direct sequencing is an appropriate alternative for deriving consensus sequences where cloning may not be possible (due to limited resources), or when cloning may be unnecessary. Here, similar research is conducted using ancient human samples where contamination is more problematic. The various difficulties with the cloning method are also addressed, along with its lack of standardization within the aDNA field.

To illustrate the variability of cloning and the lack of standardization within the aDNA field, Table 1 summarizes the amount of clones analyzed in 34 studies over a sixteen-year period (1994-2010). There is a large amount of inter-study variability with studies reporting as little as two clones to over 100 for a single amplification. This inconsistency cuts across the species studied, and the type of material used (i.e. bones, teeth, coprolites, hair, and mummified tissue).
Furthermore, it is also unclear how reviewers can evaluate consensus results when authors do not publish their clone sequences (e.g. Endicott et al., 2006; Larson et al., 2007), or when the amount of clones that were sequenced per sample is not specified (e.g., Beja-Pereira et al., 2006; Kuch et al., 2007). In these cases, it is difficult to determine the strength of the consensus sequences because readers and reviewers do not have access to the data used to support their conclusions. Table 1 also demonstrates that there is no standard for how many clones should be produced from a single sample to be considered for publication. Whether clones from multiple amplifications should be combined to form a consensus rather than as repeated verification, needs to be referenced and reported (Winters et al., 2011).

Bower and colleagues (2005) have begun to address the issue by suggesting that a minimum of 12 clones is necessary to generate a consensus sequence. They have designed an online freeware program called the “Consensus Confidence Program” (CCP), which produces a consensus of clones by calculating the probability of each nucleotide, at each position occurring at that frequency with a 70-95% confidence level. While this is perhaps the first step toward a standard methodology, there have been studies published since this recommendation that do not follow the 12 clone minimum (Table 1). It may be that this method is not possible, as it would require a large amount of systematic cloning per study, but it offers one step toward standardization of cloning practices.

This study will empirically test the standard recommendation for the practice of cloning, by comparing cloned sequences of human mtDNA to direct sequences from the same samples. A previous study (Winters et al., 2011), has shown that direct sequencing and cloning will produce the same result from aDNA samples. However, the study compared these methods on non-human, non-domesticated animal samples, and it could be argued that human samples may yield different results due to the higher risk of contamination. Based on the results of Winters and colleagues (2011), it is predicted that cloning and direct sequencing will yield the same consensus sequence, regardless of the sample type. To investigate this, DNA was extracted from ten human rib bones and amplified for 1-4 fragments within the human mtDNA D-loop (to represent HVRI) and aligned to the mtDNA Cambridge reference sequence (CRS) (Anderson et al., 1981; Andrews et al., 1999). All samples were from Native American burial sites in California, with eight from the Yukisma site (designated CA-SCL-38), and two from the El Monton site (designated CA-SCRI-333 but referred to here as "Chumash"). Results from direct sequencing and cloning were compared following a majority rules approach, and where possible, clones were analyzes in the Consensus Confidence Program (Bower et al., 2005) to determine consensus sequences.

Damage Analysis

Given that the cloning method is used to rule out error (i.e., damage) and contamination, it is also prudent to explore if the negligence of its use will increase the likelihood of publishing erroneous data. While contamination can typically be identified through repeat amplifications or by obvious differences between cloned sequences, postmortem damage may also cause nucleotide misincorporations to be erroneously displayed after aDNA amplification. These modifications manifest as nucleotide variations (i.e. "mutational changes" that are not the products of mutation), and in some cases may be misinterpreted as being derived from the DNA of the once living organism. This has led to investigations on the prevalence of postmortem damage among human mitochondrial DNA (mtDNA) (e.g. Banerjee and Brown, 2004; Gilbert et al., 2003; Meyer et al., 1999), specifically within the HVRI [nucleotide positions (nps) 16024 -16383 of the mitochondrial genome (Anderson et al., 1981; Andrews et al., 1999)], which is widely used to address genetic variation, and population histories of humans and other hominins (e.g. Caramelli et al., 2003; Gilbert et al., 2004; Kemp et al., 2007a; Ovchinnikov et al., 2000). Since one of the suggested uses for cloning is to identify damage induced errors (Cooper and Poinar, 2000), a damage analysis was also conducted in this study to test whether or not post mortem damage is randomly distributed across HVRI.

Sequence modifications that do not break the DNA strands or prevent enzymatic replication (such as hydrolytic deamination) may emerge as base variation (i.e. "mutational changes" that are not the products of mutation) in sequenced clones (Krings et al., 1997) and lead to a false consensus if the damage is most prevalent among the clones. Any nucleotide misincorporation that occurs during initial replication of the PCR may become an unambiguous base, despite being incorrect, in the resulting sequence or sequences (in the case of cloning) that are produced.

The predominance of damage in the form of transitions in aDNA studies has led to the grouping of two pairs of transitions: Type 1 (A/T→G/C) and Type 2 (C/G →T/A) (Hansen et al., 2001). However, there is debate on whether both types represent post mortem damage (Gilbert et al., 2003; Hansen et al., 2001), or if Type 1 transitions are the

artifacts of polymerase misincorporations during PCR (Hofreiter et al., 2001; Pääbo et al., 2004). Regardless, both types are typically present in aDNA sequences and further characterization is needed. Therefore, understanding and estimating the prevalence of post mortem damage in aDNA samples, along with its distribution (random or non-random) across the region of interest is applicable to authenticating results.

A non-random distribution suggests that postmortem damage is more prevalent at specific nucleotide positions over others, and conversely, a random distribution would show an equal rate (with some minimal degree of variance) across all positions. When considering the rate and distribution of damage within a sample, the risk of reporting erroneous results can be grouped into four categories (Figure 1). In any case, that damage is distributed in a non-random fashion, the risk for reporting erroneous results is higher since repeated amplifications would likely reveal damage at the same nucleotide positions and would appear unambiguous. Whenever damage is high, there is increased risk for contamination as the exogenous DNA can outcompete endogenous DNA due to the undamaged template and may continue to show up over multiple amplifications. This could be a major problem if the contaminating sequence is not easily distinguished from the sample(s) in question. Ideally, damage would be distributed randomly since repeated amplifications would help resolve what is damage and what represents the endogenous sequence. Additionally, when the rate of damage is low, the risk for contamination is also low which minimizes the risk of deriving a false consensus sequence.

One study has shown that damage does not occur randomly within the HVRI of human mtDNA (Gilbert et al., 2003), resulting in 'damage hotspots'. It is unclear whether these hotspots are the result of the structure of mtDNA (Gilbert et al., 2003; Heyer et al., 2001), environmentally induced through processes such as heat exposure (Banerjee and Brown, 2004), or other mechanisms that naturally break down DNA after death. Conversely, another study has shown that the rate of damage is random for mutational hotspots in the HVRI, and that damage was randomly distributed across the HVRI region (Kuch et al., 2007). However, this analysis was only conducted on two samples across a maximum of four clones, thus it may not be a robust example of damage distribution in this region.

A better characterization of aDNA damage is beneficial for accounting for potentially erroneous data that may result in inaccurate phylogenetic reconstructions and/or population histories. Therefore, a damage analysis was conducted to test whether or not damage is random within the HVRI region of the human mitochondrial genome, and also to test for sites with significantly higher rates of damage than would be expected randomly. Previous reports of damage in this region have shown that damage 'hotspots' exist at nucleotides within positions 16209-16356 (Gilbert et al., 2005a; Gilbert et al., 2003). Based on these findings, it was predicted that some sites within this region would yield higher rates of damage than others and that damage within the clones would be non-randomly distributed across the HVRI.

MATERIALS AND METHODS

Samples

Ancient humans burials were sampled from the Yukisma site (CA-SCL-38), an ancestral Muwekma Ohlone burial site in north Santa Clara County, California. One rib bone from each of the 252 burials was reserved for genetic analysis prior to the remaining skeletal

samples being re-interred. With approval from the Muwekma Ohlone tribe the samples were transferred to the Kemp Lab of Molecular Anthropology and Ancient DNA at Washington State University. Radiocarbon dating places deposition from 245 to at least 2205 YBP, with the majority being dated between 230-740 YBP (Gardner et al., 2011). The Chumash samples came from the El Monton site (CA-SCRI-333) on Santa Cruz Island. Samples were obtained from the Natural History Museum in London and were originally excavated by Van Valkenburgh (1933). Samples are dated between 3000-6000 YBP, with sample CHT4 being radiocarbon dated to 4190 YBP. Radiocarbon dating has not been done for the CHT6 sample but is likely within the above range.

Molecular Methods

To minimize the chance of introducing contamination, all pre-PCR methods were conducted in a laboratory dedicated to the study of low copy number (LCN) and degraded DNA, located in a separate building from the modern PCR laboratory. All surfaces within the aDNA laboratory are regularly decontaminated with a 25% diluted bleach solution (1.5% w/v). All aDNA researchers in the Kemp laboratory have been haplotyped so that all DNA sequences can be screened against obvious human contamination originating from laboratory personnel.

Extractions for the CA-SCI-38 samples were performed by Villanea (2010) and extractions for the Chumash were performed by Monroe and colleagues (2010). Both followed the protocol described by Kemp and colleagues (2007b). Each sample was first screened for the markers definitive of Native American mtDNA haplogroups A, B, C, D (Torroni et al., 1993) following Kemp et al (2007a), to help ensure the extractions were not contaminated with non-Native American mtDNA.

PCR amplification reactions contained 0.32 mM dNTPs, 1X PCR Buffer, 1.5 mM $MgCl_2$, 2.4 mM primers, 0.3 U of platinum Taq (Invitrogen^{TM}), and 1.5 μL template DNA in 15 μL reactions, or 3 μL template DNA in 30 μL reactions. Negative controls (PCR reactions to which no DNA template was added) accompanied every set of PCR reactions to monitor the presence of contaminating DNA. Primers are described by Kemp et al. (2007a).

PCR conditions were as follows: 94°C for 3 min, 60 cycles of 15 second holds at 94°C, 55°C, and 72°C, followed by a final three minute extension period at 72°C. Amplification success was determined by visualizing ~5-6 μL of the amplicons on a 6% polyacrylamide gel stained with ethidium bromide under UV light.

After screening the samples as just described, nucleotide positions (nps) 15986-16404 of the mitochondrial genome were amplified in four overlapping fragments to represent part of the HVRI. Primers D-loop 1-4 were used and are described in Kemp et al. (2007a). PCR reactions and amplifications were prepared the same as described above, except that the touch down was used (-0.1°C each round), and the annealing temperatures were 62°C for D-loop 1 and 2 primers, and 58°C for D-loop 3 and 4 primers. Products were visualized on gels (described above) in order to determine success before being submitted for direct sequencing and cloning.

After PCR amplification and gel analysis, samples were treated with *ExoI/FastAp* to remove excess primers, incorporated nucleotides, and other single stranded molecules from the PCR reaction. Five units of *ExoI* and one unit of *FastAp* was added to each sample and incubated at 37°C for 20 minutes, followed by an 80°C hold for another 20

minutes to denature the enzymes. Samples were then diluted 1:10 with dH_2O so that the volumes could be split for use in direct sequencing and clone transformation. One microliter of diluted PCR product was cloned using a TOPO® TA cloning kit and TOP10 competent cells (Invitrogen™) following manufacturer's instructions with the exception that reactions were scaled to one quarter. A minimum of 16 white colonies were selected from each sample transformation and underwent colony PCR using the M13 primers.

Amplifications for colony PCR contained 0.32 mM dNTPs, 1X PCR Buffer, 1.5 mM, 1.5 mM $MgCl_2$, 0.24 µM of each primer, 0.3 U of Platinum *Taq* polymerase (Invitrogen™), and 3.0 µL of DNA template in 15 µL reactions. These reactions were subjected to 60 cycles of PCR as follows: 3 min denaturing at 94°C, followed by 15 second holds at 94°C, 57°C, and at 72°C, with a final 3 min extension period at 72°C. Amplified products were analyzed on 2% agarose gels to identify clones containing the transformed vector. Samples containing the correct fragment were treated with *ExoI/FastAp*, using the same procedure detailed above, to purify the product. Control agar plates and PCR negatives were used to ensure cell competency, transformation efficiency and PCR amplifications free of contamination.

Amplicons were diluted between 1:10 (initial direct sequencing) and 1:50 (colony sequencing) using dH_2O. Fourteen microliters of the diluted product and 1 µL of 10 mM M13F or M13R primer were premixed and sent to the Elim Biopharmaceuticals, Inc facility in Hayward California for Sanger sequencing. Sequences were aligned to the human mtDNA Cambridge Reference Sequence (CRS) (Anderson et al., 1981; Andrews et al., 1999) using Sequencher® 4.8.

Consensus sequences were determined using a majority rules approach, choosing the clone type that represented 50% or more of the clone haplotypes. The consensus sequence was compared to the direct sequence from the same amplification. Where possible (if 12 or more clones were successfully sequenced), clone sequences were inputted into the Consensus Confidence program (Bower et al., 2005) and a consensus derived if a 95% confidence was met.

Any polymorphism that differed relative to the CRS or did not represent a mutation known for the individual haplotype was considered damage. Damage was grouped into categories of transitions and transversions to compare against expectations of Type 1 and Type 2 transitions (Hansen et al., 2001) and the rate of singleton damage, which is expected to be 4 out of 10,000 bps when Type 2 transitions are excluded (Briggs et al., 2007).

Cloned fragments were analyzed following methods described by Gilbert and colleagues (2003) except that the analysis was done for both total damage (all nucleotide positions across all clones) and unique damage (all nucleotide positions across clones with unique haplotypes). This was to compare the total number of damage hits against novel clone sequences. This follows the logic that identical damage sites occurring on several clones from the same PCR amplification may have arisen from the same template molecule (i.e. "parent" molecule), which may lead to an overestimation of damage. This follows the initial estimate for site-specific damage rates in the HVRI which were calculated using a unique sequences approach (Meyer et al., 1999). Because of the different number of amplified clones across each site, a modified relative rate of postmortem damage was calculated as $\rho_v = \mu_v/\sigma_v$, where v is a site with reference to the CRS, μ_v is the number of

damage hits observed at site v, and σ_v is the number of different clones amplified at that site.

To test for postmortem damage sites being randomly distributed across the analyzed region, the the observed substitutions were compared against the expected Poisson distribution (Aris-Brosou and Excoffier, 1996; Heyer et al., 2001). The probability that each site will have X substitutions, P(X), is $P(X) = (e^{-\lambda} \lambda^X/X!)$, and was used to calculate the expected distribution of substitutions, where λ refers to the observed density of substitutions. To estimate the expected count of sequence sites with exactly X substitutions across the length (L) of the sequence, $LP(X) = L(e^{-\lambda} \lambda^X/X!)$ was used. A G-test (goodness of fit) was then applied to the observed and expected results to determine whether the H_o that postmortem damage will be randomly distributed across the region can be rejected.

RESULTS

Cloning vs. Direct Sequencing

Out of the CA-SCL-38 samples that have been extracted for aDNA, 41 were type as belonging to one of four Native American mtDNA haplogroups (A-D) (Villanea, 2010) and 30 samples were chosen for this experiment. Of the 30 chosen, 8 samples were successfully cloned and direct sequenced from the same amplification for one or more fragments of the HVRI. The two Chumash samples were used during initial tests of the cloning procedure and were added to the pool of samples. Table 2 lists mitochondrial haplotypes for each sample.

Of the ten samples, four were analyzed at D-loop 1 fragment, three at D-loop 2, seven at D-loop 3 and eight at D-loop 4 (according to Kemp et al., 2007a), with between 3 and 22 clones sequenced for each sample fragment (Table 3). See Tables 4-6 for all direct and cloned sequences with indicated polymorphisms. A site was designated “N” if the sequence chromatogram showed competition between two bases.

With the exception of three fragments, none of the samples showed any difference between the direct sequence, majority rules consensus, and when possible, the consensus determined by the CCP (Table 3). It was not possible to obtain 12 clones for all fragments across all samples and so the CCP could not be used in all cases. A consensus for two fragments (B001 – D loop 1 and CHT4 – D loop 3) could not be made as the clones did not have a sequence type that held a majority over all clones sequenced. Where consensus sequences could be generated, all samples demonstrated the appropriate haplotype substitutions with the exception of CHT6. Notably, while CHT6 was sequenced to confirm the A haplotype, the sequence results were for a haplotype W individual. The W haplotype does not exist within Native American populations, and no researcher within the Kemp lab carries this haplotype. The sequences for this sample are attributed to contamination from reagents and/or lab disposables, but since all clones represent this haplotype, the clones were used for this study. One fragment (B048 – D loop 1) showed a discrepancy between the direct sequence and the consensus determined by the clones and CCP, with the direct sequence showing an N at position 16089, and the clones showing a majority of adenine at that position over guanine.

Damage Analysis

The cloned data followed the expected pattern of miscoding lesions representing singleton sites of damage throughout the sequences (Briggs et al., 2007; Gilbert et al., 2007). When including damage across all clones, the majority of the damaged sites were C→T transitions (28 hits, 46% of damage), and G→A (13 hits, 21%), which are both indicative of deamination (Hofreiter et al., 2001). These occurred at a rate much higher than other transitions and transversions (see Figure 2): 1) A→G transitions (11 hits, 18%), 2) T→C (6 hits, 9%), and 3) T→A, G→C, and A→C transversions represent 2% of the damage each. When analyzing damage across unique clones, only the C/G→T/A transitions are reduced (Figure 2), with C→T transitions maintaining the majority (23 hits, 49%), but G→A transitions dropping to 4 hits (8%). In either case, neither total or unique damage exceed expectations for singleton rates of nucleotide misincorporations as 20 hits of damage over 94,860 bps (255 clones across 372 bps) is less than the expected 4 hits over 10,000 bps (Briggs et al., 2007). The overall bias towards transitions, and specifically Type 2 transitions (C/G→T/A) is as expected and has been observed in previous aDNA studies (Gilbert et al., 2005a; Gilbert et al., 2003; Hansen et al., 2001).

Total and unique counts of damage are shown in Figures 3 and 4, with and without the inclusion of N sites. Nucleotide position 16266 shows a high count of damage across both (8 hits for total, 5 hits for unique), and position 16089 shows a high count for total damage (10 hits) but only counts for 2 hits within unique damage. While position 16290 has 3 hits across both categories, this is considered the second highest count within unique damage. The remaining 36 sites show 1-3 hits for total damage, or 1-2 hits for unique damage. The relative rates of damage for both total and unique damage are compared in Figure 5. As shown, the relative rate for position 16089 is the largest with total damage representing 25% of the sequenced clones, and unique damage representing 14.3%. This corresponds with position 16089 showing a high total damage count (Figure 3).

The null hypothesis (H_o) that postmortem damage is randomly distributed cannot be rejected for the P(X) for both total damage ($G = 0.26474_{df=10}$, $P = 1$) and unique damage ($G = 0.05443_{df=5}$, $P = 1$), demonstrating that sites are damaged randomly across the region (Figure 6). However, when comparing observed counts to the expected counts for LP(X), total damage did show a significant G value ($G = 100.5932_{df=10}$, $P = 0.0001$), but unique damage did not ($G = 6.4992_{df=5}$, $P = 0.2606$). The significance for total damage was thought to be attributed to the two sites that show overrepresentation of damage (nps 16089 and 16266) since the observation of 8 and 10 hits of damage at one nucleotide far exceeds expectations. To determine if this was the case, another comparison was done that excluded the two sites that showed the high damage. The results were not significant ($G = 5.39824_{df=3}$, $P = 0.1449$) and support that damage is randomly distributed when overrepresented (non-unique) damage is excluded.

DISCUSSION

While a previous study has already demonstrated that directly sequencing aDNA of non-domesticated animals will provide the same consensus as cloning (Winters et al., 2011) here it is shown that these two methods will yield the same data when attempting to compile a consensus sequence from humans as well (Table 3). This does not imply that cloning is an unnecessary tool for aDNA research as it is necessary for the scope of some research questions. Cloning can be done in order to 'find' the sequence of interest when contamination makes it difficult to retrieve endogenous molecules (Gilbert et al.,

2008; Krause et al., 2007). Cloning is also necessary for reconstructing ancient diets (Poinar et al., 1998) and resolving mixed sequences (Willerslev et al., 2007). Cloning has also been used to identify patterns of nucleotide misincorporations so that the rates can be applied to high-throughput analysis of ancient genomes to reduce underestimation of damage (Stiller et al., 2006). However, when the focus is to retrieve the consensus sequence of an individual sample, this is further evidence that cloning may be superfluous over direct sequencing and should not be considered a requirement for publication.

Although molecular damage in aDNA molecules can lead to the incorrect identification of consensus sequences on some occasions (Hofreiter et al., 2001), it is unlikely to be the case here. The fact that all clones demonstrated the correct haplotype substitution markers (see Table 3) in conjunction with the presence of damage (except in the case of one clone in the fourth fragment of sample B038 and 10 clones in the first fragment of sample B048) suggests that the consensus sequences represent the endogenous mtDNA for these samples. In the case of sample B001 – D loop 1, the direct sequence contained N sites that might suggest competing base pairs at those positions. In theory, cloning this product would resolve the ratio of endogenous to exogenous molecules (Cooper and Poinar, 2000; Pääbo et al., 2004), but only three clones were retrieved for this sample. As each clone differed, there was no confidence in determining a consensus despite previous studies that have published a consensus sequence when this was the case (Handt et al., 1996).

Sample B038 – D loop 4 contained one clone that did not possess the polymorphisms expected of a haplogoup D individual even though the remaining 12 clones did so. While this shows some form of contamination or damage at the defining mutation which caused it to revert to the ancestral state that likely exists within the sample, the remaining clones show what is expected, and since no researcher within the Kemp lab is of the D haplotype, the results make sense. More interesting is sample B038 – D loop 1, where 5 clones show sporadic N sites throughout the region. The direct sequence does not reflect this, and so the N sites are likely the result of errors introduced during the cloning process or subsequent PCR and sequencing. If a researcher only had those 11 clones from which they were to derive a consensus, it may not be considered a reliable outcome in comparison to the results of the direct sequence.

Sample B048 – D loop 1 showed 10 clones with an adenine at position 16089, and 2 clones with the expected guanine for a haplotype D individual. While it might be argued that the adenine represents the template nucleotide for this individual, 16089 is not a variable site among human mtDNA according to the Human Mitochondrial Genome Database (Ingman and Gyllensten, 2006) and mitomap (Mitomap, 2011). These databases contain records of thousands of human mitochondrial genomes and the fact that not one reference found polymorphisms at this site suggests that the adenine is the result of damage. The direct sequence shows an N site at this position and the chromatogram reveals that there is competition between the guanine and adenine nucleotides (see Figure 7). It is therefore likely that, by chance alone, the clone vectors picked up a large number of the damaged molecules, or the colonies that contained the molecule were picked more than colonies that contained the undamaged molecule. Again, without knowledge of the direct sequence, the consensus for this sequence would initially look like a novel mutation, and in either case re-amplification or re-extraction of the sample will need to be done to authenticate the results. This also demonstrates, that

in some cases, taking the majority rules of a group of clones may not represent the sequence endogenous to the sample.

The cloning method is not without methodological complications, even when using a kit. While the initial goal of this study was to sequence four overlapping fragments of the HVRI for 30 samples, only a fraction of this was achieved due to complications and failure during cloning. In many cases transformation efficiency was low; either due to degradation of the adenine overhang of the PCR product (which did not allow vector ligation), or failure of the vector to pick up the DNA fragment or transform into the cells. Many transformations yielded less than 12 useable colonies (or none at all), which made analysis impossible. It is likely that other researchers have experienced complications and inefficiency using this practice. In this case, resources would be better devoted to direct sequencing the same amount of samples multiple times and using the replicates to authenticate their data. In all cases, cloning gave no added confirmation of the endogenous DNA then the direct sequence. It did however introduce errors and damage that is not seen in the direct sequence.

The damage reported in this study does not agree with the result of being non-randomly distributed as was found in previous reports in this region of the mitochondrial genome (Gilbert et al., 2003). Analyses for both total and unique damage showed that there was a random distribution of damage throughout the region. The only exceptions to this were at np 16089, where 10 G→A transitions were viewed across one sample (B048), and at np 16266, where 8 C→T transitions were viewed across the clones of three samples (CHT4, B009 and B038). The increased rate of total damage at 16089 appears overrepresented as it comes from one sample. When the damage is reduced to unique haplotypes, the damage only represents two hits. The C→T transition at 16266 is common within the Human Mitochondrial Genome Database (Ingman and Gyllensten, 2006), showing 34 other samples in 9 studies that recorded this polymorphism (but this is compared to 1820 samples that didn't show the transition). However, 16266 is represented by 5 unique haplotypes across three samples and may be indicative of a damage hotspot, but as this site is not considered a mutational hotspot (Meyer et al., 1999), it is unlikely that the transitions seen here are representative of the sample. The CHT4 sample appears to be particularly degraded at this fragment with seven unique clone sequences that are contributing to the damage rate at np 16266, Some of this damage could be attributed to jumping PCR events across daughter amplified molecules (Pääbo, 1989) or the sample itself may be heteroplasmic. This is also one of the few samples where a consensus could not be reached and further suggests that this may be implicit of a highly degraded sample, or that by chance alone, the molecules that were amplified during PCR represented a very heterogeneous pool of chemically modified molecules. Subsequent direct sequences (N=3) of this sample have shown an N (with C/T competition) at this position, but have not shown polymorphisms at sites other than would be expected for the haplotype. The transitions at np 16266 should be considered as damage, especially since they represent a C→T transition, the most common form of deamination (Briggs et al., 2007; Hofreiter et al., 2001). Because of these reasons, the two sites were excluded from the LP(X) estimate as they appeared to give a false significance to damage being non-random in the analyzed region. Regardless, when analyzing P(X) and LP(X) for unique damage, which represents a more conserved estimate of damage because only novel clone haplotypes are considered where clones of the same type could stem from the same parent template molecule, there was no significant difference between the expected and observed rates of damage.

While the random distribution does not agree with the results found by Gilbert and colleagues (2003), it does agree with the other study on damage rate, indicating that damage sites in aDNA are not preferentially occurring at mutational hotspots within the HVRI (Kuch et al., 2007). Although three sites correlated with mutational hotspots (nps 16166, 16293, 16362), they only showed one hit of damage and therefore do not appear preferentially damaged. There was no instance where a highly damaged nucleotide position (more than two hits) correlated with a mutational hotspot (as defined by Meyer et al., 1999).

Differences in analysis and choice of samples may attribute to these findings. For example, Gilbert and colleagues (2003) used 34 ancient human samples from Britain, Denmark, and Greenland, with a range of 2-80 clones per individual. Comparing the results of this study, it did not meet the same measurement of damage as Gilbert and colleagues (2003), both in the number of individuals, and the amount of clones used. Additionally, this study measured variation in damage rate across the entire region instead of dividing it into two parts, as was done in the comparative study to adjust for the fact that they amplified the region between nps16209-16356 more often (Gilbert et al., 2003).

CONCLUSION

This study shows that, even in the case of ancient human DNA, utilizing direct sequencing to obtain consensus sequences is a viable alternative to cloning. Reviewers should not consider the process of cloning itself as an authentication process, and should instead review the data to ensure it makes sense. While the recommendations of Cooper and Poinar (2000) should not be dismissed outright, as many of the criteria help to reduce contamination and dubious claims, publication of research should not be dictated by a methods checklist (Gilbert et al., 2005b; Kemp and Smith, 2010; Winters et al., 2011). Researchers should be as explicit as possible when describing their methods so that reviewers and readers alike can use the data to determine authenticity.

This study also supports that damage is random and not strongly correlated with mutational hotspots. This suggests that the risk of identifying erroneous sequences is low, since damage is not expected to be overrepresented. The characterization of post mortem damage still needs more study across this region to resolve the discrepancies between studies reported to date and should strive to include a wide range of samples and haplotypes. This could determine if damage is distributed differently based on the environment surrounding the samples after death or if some populations experience a different distribution due to the structure of the mtDNA in this region (Gilbert et al., 2003; Heyer et al., 2001).

ACKNOWLEDGMENTS

This project was supported by Award No. 2008-DN-BX-K008 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings, and conclusions or recommendations expressed in this publication/program/exhibition are those of the author(s) and do not necessarily reflect those of the Department of Justice. Thanks to Rosemary Cambra, Silvia Gonzalez, and Alan Leventhal for providing the samples studied here.

WORKS CITED

Anderson S, Bankier A, Barrell BG, De Bruijn M, Coulson A, Drouin J, Eperon I, Nierlich D, Roe B, and Sanger F. 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457-465.

Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, and Howell N. 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nature genetics 23(2):147.

Aris-Brosou S, and Excoffier L. 1996. The impact of population expansion and mutation rate heterogeneity on DNA sequence polymorphism. Molecular biology and evolution 13(3):494-504.

Banerjee M, and Brown TA. 2004. Non-random DNA damage resulting from heat treatment: implications for sequence analysis of ancient DNA. Journal of archaeological science 31(1):59-63.

Beja-Pereira A, Caramelli D, Lalueza-Fox C, Vernesi C, Ferrand N, Casoli A, Goyache F, Royo LJ, Conti S, and Lari M. 2006. The origin of European cattle: evidence from modern and ancient DNA. PNAS 103(21):8113-8118.

Binladen J, Wiuf C, Gilbert MT, Bunce M, Barnett R, Larson G, Greenwood AD, Haile J, Ho SY, Hansen AJ and others. 2006. Assessing the fidelity of ancient DNA sequences amplified from nuclear genes. Genetics 172(2):733-741.

Bouwman AS, and Brown TA. 2005. The limits of biomolecular palaeopathology: ancient DNA cannot be used to study venereal syphilis. Journal of Archaeological Science 32(5):703-713.

Bower MA, Spencer M, Matsumura S, Nisbet RER, and Howe CJ. 2005. How many clones need to be sequenced from a single forensic or ancient DNA sample in order to determine a reliable consensus sequence? Nucleic Acids Research 33(8):2549-2556.

Briggs AW, Stenzel U, Johnson PLF, Green RE, Kelso J, Prüfer K, Meyer M, Krause J, Ronan MT, Lachmann M and others. 2007. Patterns of damage in genomic DNA sequences from a Neandertal. Proceedings of the National Academy of Sciences 104(37):14616.

Caramelli D, Lalueza-Fox C, Vernesi C, Lari M, Casoli A, Mallegni F, Chiarelli B, Dupanloup I, Bertranpetit J, Barbujani G and others. 2003. Evidence for a genetic discontinuity between Neandertals and 24,000-year-old anatomically modern Europeans. Proc Natl Acad Sci U S A 100(11):6593-6597.

Cooper A, and Poinar HN. 2000. Ancient DNA: do it right or not at all. Science 289(5482):1139.

Dissing J, Kristinsdottir MA, and Friis C. 2008. On the elimination of extraneous DNA in fossil human teeth with hypochlorite. Journal of archaeological science 35(6):1445-1452.

Endicott P, Metspalu M, Stringer C, Macaulay V, Cooper A, and Sanchez JJ. 2006. Multiplexed SNP typing of ancient DNA clarifies the origin of andaman mtDNA haplogroups amongst south Asian tribal populations. PLoS One 1(1):e81.

Gardner KS, Leventhal A, Cambra R, Tribe MO, Bartelink EJ, and Martinez A. 2011. Mothers and infants in the prehistoric Santa Clara Valley: What stable isotopes tell us about ancestral Ohlone weaning practices. SCA Proceedings 25.

Gilbert M, Shapiro B, Drummond A, and Cooper A. 2005a. Post-mortem DNA damage hotspots in Bison (Bison bison) provide evidence for both damage and mutational hotspots in human mitochondrial DNA. Journal of archaeological science 32(7):1053-1060.

Gilbert MT, Menez L, Janaway RC, Tobin DJ, Cooper A, and Wilson AS. 2006. Resistance of degraded hair shafts to contaminant DNA. Forensic Sci Int 156(2-3):208-212.
Gilbert MT, Wilson AS, Bunce M, Hansen AJ, Willerslev E, Shapiro B, Higham TF, Richards MP, O'Connell TC, Tobin DJ and others. 2004. Ancient mitochondrial DNA from hair. Curr Biol 14(12):R463-464.
Gilbert MTP, Bandelt HJ, Hofreiter M, and Barnes I. 2005b. Assessing ancient DNA studies. Trends in Ecology & Evolution 20(10):541-544.
Gilbert MTP, Binladen J, Miller W, Wiuf C, Willerslev E, Poinar H, Carlson JE, Leebens-Mack JH, and Schuster SC. 2007. Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis. Nucleic Acids Research 35(1):1-10.
Gilbert MTP, Jenkins DL, Götherstrom A, Naveran N, Sanchez JJ, Hofreiter M, Thomsen PF, Binladen J, Higham TFG, and Yohe RM. 2008. DNA from pre-Clovis human coprolites in Oregon, North America. Science 320(5877):786.
Gilbert MTP, Willerslev E, Hansen AJ, Barnes I, Rudbeck L, Lynnerup N, and Cooper A. 2003. Distribution patterns of postmortem damage in human mitochondrial DNA. The American Journal of Human Genetics 72(1):32-47.
Green RE, Malaspinas AS, Krause J, Briggs AW, Johnson PLF, Uhler C, Meyer M, Good JM, Maricic T, and Stenzel U. 2008. A complete Neandertal mitochondrial genome sequence determined by high-throughput sequencing. Cell 134(3):416-426.
Haak W, Forster P, Bramanti B, Matsumura S, Brandt G, Tanzer M, Villems R, Renfrew C, Gronenborn D, Alt KW and others. 2005. Ancient DNA from the first European farmers in 7500-year-old Neolithic sites. Science 310(5750):1016-1018.
Handt O, Krings M, Ward RH, and Pääbo S. 1996. The retrieval of ancient human DNA sequences. American Journal of Human Genetics 59(2):368.
Handt O, Richards M, Trommsdorff M, Kilger C, Simanainen J, Georgiev O, Bauer K, Stone A, Hedges R, and Schaffner W. 1994. Molecular genetic analyses of the Tyrolean Ice Man. Science 264(5166):1775.
Hansen AJ, Willerslev E, Wiuf C, Mourier T, and Arctander P. 2001. Statistical evidence for miscoding lesions in ancient DNA templates. Molecular biology and evolution 18(2):262-265.
Helgason A, Lalueza-Fox C, Ghosh S, Sigurethardottir S, Sampietro ML, Gigli E, Baker A, Bertranpetit J, Arnadottir L, Thornorsteinsdottir U and others. 2009. Sequences from first settlers reveal rapid evolution in Icelandic mtDNA pool. PLoS Genet 5(1):e1000343.
Heyer E, Zietkiewicz E, Rochowski A, Yotova V, Puymirat J, and Labuda D. 2001. Phylogenetic and familial estimates of mitochondrial substitution rates: study of control region mutations in deep-rooting pedigrees. The American Journal of Human Genetics 69(5):1113-1126.
Hofreiter M, Capelli C, Krings M, Waits L, Conard N, Munzel S, Rabeder G, Nagel D, Paunovic M, Jambresic G and others. 2002. Ancient DNA analyses reveal high mitochondrial DNA sequence diversity and parallel morphological evolution of late pleistocene cave bears. Mol Biol Evol 19(8):1244-1250.
Hofreiter M, Jaenicke V, Serre D, von Haeseler A, and Pääbo S. 2001. DNA sequence from mulitple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. Nucleic Acids Research 29(23):4793-4799.
Ingman M, and Gyllensten U. 2006. mtDB: Human Mitochondrial Genome Database, a resource for population genetics and medical sciences. Nucleic Acids Res 34:D749-D751.

Karanth KP, Delefosse T, Rakotosamimanana B, Parsons TJ, and Yoder AD. 2005. Ancient DNA from giant extinct lemurs confirms single origin of Malagasy primates. Proc Natl Acad Sci U S A 102(14):5090-5095.
Kemp B, Malhi R, McDonough J, Bolnick D, Eshleman J, Rickards O, Martinez-Labarga C, Johnson J, Lorenz J, and Dixon E. 2007a. Genetic analysis of early Holocene skeletal remains from Alaska and its implications for the settlement of the Americas. American Journal of Physical Anthropology 132(4):605.
Kemp BM, Monroe C, and Smith DG. 2007b. Extraction and analysis of DNA from archaeological specimens. ACS Publications. p 78-98.
Kemp BM, and Smith DG. 2010. Ancient DNA Methodology: "Thoughts from Brian M. Kemp and David Glenn Smith on "Mitochondrial DNA of Protohistoric Remains of an Arikara Population from South Dakota". Human Biology 82(2):227-238.
Kim JH, Oh JH, Song JH, Jeon JT, Han SH, Jung YH, and Oh MY. 2005. Molecular genetic analysis of ancient cattle bones excavated from archaeological sites in Jeju, Korea. Mol Cells 20(3):325-330.
Krause J, Orlando L, Serre D, Viola B, Prufer K, Richards MP, Hublin JJ, Hanni C, Derevianko AP, and Paabo S. 2007. Neanderthals in central Asia and Siberia. Nature 449(7164):902-904.
Krings M, Geisert H, Schmitz RW, Krainitzki H, and Paabo S. 1999. DNA sequence of the mitochondrial hypervariable region II from the neandertal type specimen. Proc Natl Acad Sci U S A 96(10):5581-5585.
Krings M, Stone A, Schmitz RW, Krainitzki H, Stoneking M, and Paabo S. 1997. Neandertal DNA sequences and the origin of modern humans. Cell 90(1):19-30.
Kuch M, Gröcke DR, Knyf MC, Gilbert MTP, Younghusband B, Young T, Marshall I, Willerslev E, Stoneking M, and Poinar H. 2007. A preliminary analysis of the DNA and diet of the extinct Beothuk: A systematic approach to ancient human DNA. American Journal of Physical Anthropology 132(4):594-604.
Kuhn TS, McFarlane KA, Groves P, Mooers AO, and Shapiro B. 2010. Modern and ancient DNA reveal recent partial replacement of caribou in the southwest Yukon. Mol Ecol 19(7):1312-1323.
Lari M, Rizzi E, Milani L, Corti G, Balsamo C, Vai S, Catalano G, Pilli E, Longo L, Condemi S and others. 2010. The microcephalin ancestral allele in a Neanderthal individual. PLoS One 5(5):e10648.
Larson G, Albarella U, Dobney K, Rowley-Conwy P, Schibler J, Tresset A, Vigne JD, Edwards CJ, Schlumbaum A, and Dinu A. 2007. Ancient DNA, pig domestication, and the spread of the Neolithic into Europe. Proceedings of the National Academy of Sciences 104(39):15276.
Loreille O, Orlando L, Patou-Mathis M, Philippe M, Taberlet P, and Hanni C. 2001. Ancient DNA analysis reveals divergence of the cave bear, Ursus spelaeus, and brown bear, Ursus arctos, lineages. Curr Biol 11(3):200-203.
Malmström H, Storå J, Dalén L, Holmlund G, and Götherström A. 2005. Extensive human DNA contamination in extracts from ancient dog bones and teeth. Molecular biology and evolution 22(10):2040-2047.
Meyer S, Weiss G, and Von Haeseler A. 1999. Pattern of nucleotide substitution and rate heterogeneity in the hypervariable regions I and II of human mtDNA. Genetics 152(3):1103-1110.
Mitomap. 2011. A human mitochondrial genome database. Center for Molecular Medicine, Emory University, Atlanta, GA, USA Internet address: http://wwwmitomaporg.
Monroe C, Gonzalez S, Johnson J, Kruszynski R, and Kemp BM. 2010. Ancient DNA Analysis of Prehistoric Burials from the Santa Barbara Channel Region Paper

presented at the 75th annual meeting of the Society for American Archaeology, St Louis, MO.
Monsalve MV, Stone AC, Lewis CM, Rempel A, Richards M, Straathof D, and Devine DV. 2002. Brief communication: molecular analysis of the Kwaday Dan Ts'finchi ancient remains found in a glacier in Canada. Am J Phys Anthropol 119(3):288-291.
Orlando L, Darlu P, Toussaint M, Bonjean D, Otte M, and Hanni C. 2006. Revisiting Neandertal diversity with a 100,000 year old mtDNA sequence. Curr Biol 16(11):R400-402.
Orlando L, Leonard JA, Thenot A, Laudet V, Guerin C, and Hanni C. 2003. Ancient DNA analysis reveals woolly rhino evolutionary relationships. Mol Phylogenet Evol 28(3):485-499.
Ovchinnikov IV, Götherström A, Romanova GP, Kharitonov VM, Lidén K, and Goodwin W. 2000. Molecular analysis of Neanderthal DNA from the northern Caucasus. Nature 404(6777):490-493.
Pääbo S. 1989. Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proceedings of the National Academy of Sciences 86(6):1939.
Pääbo S, Poinar H, Serre D, Jaenicke-Després V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, and Hofreiter M. 2004. Genetic analyses from ancient DNA. Annual Review of Genetics 38:645-679.
Poinar H, Kuch M, McDonald G, Martin P, and Paabo S. 2003. Nuclear gene sequences from a late Pleistocene sloth coprolite. Current Biology 12(13):1150-1152.
Poinar HN, Hofreiter M, Spaulding WG, Martin PS, Stankiewicz BA, Bland H, Evershed RP, Possnert G, and Paabo S. 1998. Molecular coproscopy: Dung and diet of the extinct ground sloth Nothrotheriops shastensis. Science (Washington D C) 281(5375):402-406.
Salamon M, Tuross N, Arensburg B, and Weiner S. 2005. Relatively well preserved DNA is present in the crystal aggregates of fossil bones. Proc Natl Acad Sci U S A 102(39):13783-13788.
Serre D, Hofreiter M, and Pääbo S. 2004. Mutations induced by ancient DNA extracts? Molecular biology and evolution 21(8):1463-1467.
Stiller M, Green RE, Ronan M, Simons J, Du L, He W, Egholm M, Rothberg J, Keates S, and Ovodov N. 2006. Patterns of nucleotide misincorporations during enzymatic amplification and direct large-scale sequencing of ancient DNA. Proceedings of the National Academy of Sciences 103(37):13578.
Torroni A, Schurr TG, Cabell MF, Brown MD, Neel JV, Larsen M, Smith DG, Vullo CM, and Wallace DC. 1993. Asian affinities and continental radiation of the four founding Native American mtDNAs. American Journal of Human Genetics 53(3):563.
VanValkenburgh RF. 1933. Archaeological Excavations on Frazier Point, Santa Cruz Island, California, Redondo, California.
Villanea FA. 2010. Evolution of the ABO blood group locus in pre-Columbian Native Americans. Pullman: Washington State University.
Willerslev E, Cappellini E, Boomsma W, Nielsen R, Hebsgaard MB, Brand TB, Hofreiter M, Bunce M, Poinar HN, Dahl-Jensen D and others. 2007. Ancient biomolecules from deep ice cores reveal a forested southern Greenland. Science 317(5834):111-114.
Winters M, Barta JL, Monroe C, and Kemp BM. 2011. To Clone or Not To Clone: Method Analysis for Retrieving Consensus Sequences In Ancient DNA Samples. PLoS One 6(6):e21247.

Table 1 Selection of studies over a 16-year period that utilized cloning in the study of aDNA

		Number of Clones Sequenced											
Study	**Comparison to Direct Sequence?**	**2**	**3**	**4-5**	**6-8**	**9-11**	**12-20**	**21-40**	**41-60**	**61-80**	**81-100**	**101+**	**Species/Samples**
(Handt et al., 1994)	N						X						Human (Tyrolean Iceman)
(Handt et al., 1996)	N		X	X	X								Human
(Krings et al., 1997)	N			X	X	X	X						Neanderthal
(Poinar et al., 1998)	N				X	X	X						Ground Sloth
(Krings et al., 1999)	N				X	X	X						Neanderthal
(Ovchinnikov et al., 2000)	Y		X	X									Neanderthal
(Hofreiter et al., 2001)	N				X	X							Cave Bear
(Loreille et al., 2001)	Y		X	X	X								Cave Bear and Brown Bear
(Hofreiter et al., 2002)	N		X	X	X	X	X						Cave bear
(Monsalve et al., 2002)	Y			X	X								Human
(Caramelli et al., 2003)	N			X	X	X							Human
(Gilbert et al., 2003)	N	X		X	X	X	X	X	X	X			Human
(Orlando et al., 2003)	N	X	X	X	X								Woolly rhinoceros
(Poinar et al., 2003)	N				X	X	X						Sloth
(Gilbert et al., 2004)	N			X	X	X	X						Human
(Serre et al., 2004)	N			X	X	X	X						Human, Cave Bear and Brown Bear
(Bouwman and Brown, 2005)	Y			X									Humans, Syphilis
(Gilbert et al., 2005a)^	N				X								Bison
(Haak et al., 2005)	N			X	X	X	X						Human
(Kim et al. 2005)	N		X										Cows
(Karanth et al. 2005)	N		X	X									Lemurs
(Malmström et al. 2005)	N								X				Human, Dog
(Salamon et al. 2005)	N			X	X								Cat, Penguin, Human

Table 1 (cont'd)

		Number of Clones Sequenced											
Study	**Comparison to Direct Sequence?**	**2**	**3**	**4-5**	**6-8**	**9-11**	**12-20**	**21-40**	**41-60**	**61-80**	**81-100**	**101+**	**Species/Samples**
(Binladen et al. 2006)	Y			X		X							Woolly Rhinoceros, Lion, Pig, Moa
(Gilbert et al. 2006)	N					X			X	X			Human
(Orlando et al. 2006)	N				X		X						Neanderthal
(Stiller et al. 2006)	N			X	X	X	X						Dog
(Krause et al. 2007)	N		X		X		X				X	X	Neanderthal
(Kuch et al. 2007)*	N			X	X								Human
(Dissing et al. 2008)	N			X	X	X							Human
(Green et al. 2008)	N											X	Neanderthal
(Helgason et al. 2009)	N	X	X	X	X	X	X	X	X	X			Human
(Kuhn et al. 2010)	Y					X							Caribou
(Lari et al. 2010)	N						X	X					Neanderthal

This table was adopted from Winters et al (2011) with more studies added. Categories for number of clones were arbitrarily chosen.
^estimated number of clones based on an average (679clones/81 samples) (Gilbert et al. 2005a)
*estimated number of clones from Figure 3 (Kuch et al. 2007)

Table 2 Mitochondrial haplotypes for the CA-SCl-38 and Chumash samples

Sample	**B001**	**B009**	**B023**	**B024**	**B026**	**B038**	**B048**	**B064**	**CHT4**	**CHT6**
Haplotype	A	D	B	D	D	D	D	C	A	A*

*CHT6 was tentatively haplotyped as A but was not confirmed

Table 3 Results from the sequencing and consensus analysis of all samples. Samples were sequenced from nucleotide positions 16011-16382 using four overlapping fragments of the human mitochondrial D-loop, relative to the human mtDNA Cambridge Reference Sequence (CRS) (Anderson et al. 1981; Andrews et al. 1999). Polymorphisms listed are relative to the CRS, with polymorphisms that represent damage/error listed in bold, and polymorphisms that represent haplotype markers not listed in bold. CCP refers to the consensus sequence determined by the Consensus Confidence Program (Bower et al. 2005). For the direct sequence category, "MATCH" indicates that the sequence is the same as the majority rules determined by the clones.

Sample	Majority Rules	CCP	Direct Sequence
B001 - D loop 1	N/A	N/A	**16076N**, **16080N**, **16089N**, 16111T
B001 - D loop 2	16223T	16223T	MATCH
B001 - D loop 4	16290T, 16319A, 16362C	16290T, 16319A, 16362C	MATCH
B009 - D loop 3	16223T, 16325C	N/A	MATCH
B009 - D loop 4	16325C, 16362C	16325C, 16362C	MATCH
B023 - D loop 1	16126C	16126C	MATCH
B023 - D loop 3	16217C	N/A	MATCH
B023 - D loop 4	Reference	N/A	MATCH
B024 - D loop 2	16223T	16223T	MATCH
B024 - D loop 3	16223T, 16325C	N/A	MATCH
B024 - D loop 4	16325C, 16362C	16325C, 16362C	MATCH
B026 - D loop 4	16325C, 16362C	N/A	MATCH
B038 - D loop 1	Reference	N/A	MATCH
B038 - D loop 3	16223T, 16325C	16223T, 16325C	MATCH
B038 - D loop 4	16325C, 16362C	16325C, 16362C	MATCH
B048 - D loop 1	**16089A**	**16089A**	**16098N**
B048 - D loop 2	16223T	N/A	MATCH
B048 - D loop 4	16325C, 16362C	N/A	MATCH
B064 - D loop 3	16223T, 16298C, 16325C, 16327T	16223T, 16298C, 16325C, 16327T	MATCH

Table 3 Cont'd

B064 - D loop 4	16298C, 16325C, 16327T	16298C, 16325C, 16327T	MATCH
CHT4 - D loop 3	N/A	N/A	16223T, 16263A, 16290T, 16319A
CHT6 - D loop 3	16223T, 16292T	16223T, 16292T	MATCH

Table 4 Direct sequencing and clone results for the first and second fragment of the human mtDNA HVRI (positions 16011-16131 and 16127-16229). Letters in red represent damage or error (N sites). With the exception of B001, the first clone sequence represents the consensus.

	16019	16042	16051	16052	16053	16054	16055	16076	16080	16082	16085	16089	16092	16094	16103	16104	16111	16116	16126	16133	16150	16166	16192	16194	16207	16211	16223
Reference	C	G	A	C	C	A	C	C	A	C	C	G	T	T	A	C	C	A	T	C	C	A	C	A	A	C	C
B001 - Direct 1st	.	.	.	.	.	.	.	N	N	.	.	N	.	.	.	.	T	.	.								
B001 - Clones (1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.								
B001 - Clones (1)	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.								
B001 - Clones (1)	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.								
B023 - Direct 1st	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C								
B023 - Clones (13)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C								
B023 - Clones (1)	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C								
B038 - Direct 1st	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.								
B038 - Clones (6)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.								
B038 - Clones (1)	N	.	.	N	N	.	N	.	.	N	N	.	.	.	.	.	.	.	.								
B038 - Clones (1)	.	.	N	N	N	N	N	.	.	.	.	.	.	.	.	.	.	.	.								
B038 - Clones (1)	.	.	.	.	N	N	.	.	.	.	.	.	.	.	.	.	.	.	.								
B038 - Clones (1)	.	.	.	.	.	.	.	.	.	.	N	.	.	N	N	N	.	.	.								
B038 - Clones (1)	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.								
B048 - Direct 1st	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.								
B048 - Clones (9)	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.								
B048 - Clones (2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.								
B048 - Clones (1)	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	G	.								

Table 4 cont'd

	16019	16042	16051	16052	16053	16054	16055	16076	16080	16082	16085	16089	16092	16094	16103	16104	16111	16116	16126	16133	16150	16166	16192	16194	16207	16211	16223
Reference	C	G	A	C	C	A	C	C	A	C	C	G	T	T	A	C	C	A	T	C	C	A	C	A	A	C	C
B001 - Direct 2nd																				.	.	.	.	.	.	.	T
B001 - Clones (12)																				.	.	.	.	.	.	.	T
B001 - Clones (1)																				T	.	.	.	.	.	.	T
B001 - Clones (1)																				.	.	.	.	G	.	.	T
B024 - Direct 2nd																				.	.	.	.	.	.	.	T
B024 - Clones (11)																				.	.	.	.	.	.	.	T
B024 - Clones (2)																				.	T	.	.	.	.	.	T
B048 - Direct 2nd																				.	.	.	.	.	.	.	T
B048 - Clones (8)																				.	.	.	.	.	.	.	T
B048 - Clones (1)																				.	.	G	.	.	.	.	T
B048 - Clones (1)																				.	.	.	.	.	.	T	T

Table 5 Direct sequencing and clone results for the third fragment of the human mtDNA HVRI (positions 16210-16330). Letters in red represent damage or error (N sites). With the exception of CHT4, the first clone sequence represents the consensus.

	16217	16223	16229	16238	16258	16259	16263	16264	16266	16270	16273	16283	16287	16289	16290	16292	16295	16298	16305	16306	16319	16325	16327
Reference	T	C	T	T	A	C	T	C	C	C	G	A	C	A	C	C	C	T	A	C	G	T	C
B009 - Direct 3rd	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
B009 - Clones (6)	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
B009 - Clones (2)	.	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.
B009 - Clones (1)	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
B023 - Direct 3rd	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B023 - Clones (4)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B023 - Clones (1)	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
B024 - Direct 3rd	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
B024 - Clones (8)	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
B024 - Clones (1)	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
B038 - Direct 3rd	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
B038 - Clones (10)	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
B038 - Clones (1)	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	C	.
B038 - Clones (1)	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.
B064 - Direct 3rd	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	T
B064 - Clones (11)	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	T
B064 - Clones (1)	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	T
B064 - Clones (1)	.	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	T

Table 5 cont'd

	16217	16223	16229	16238	16258	16259	16263	16264	16266	16270	16273	16283	16287	16289	16290	16292	16295	16298	16305	16306	16319	16325	16327
Reference	T	C	T	T	A	C	T	C	C	C	G	A	C	A	C	C	C	T	A	C	G	T	C
CHT4 - Direct 3rd	.	T	.	.	.	.	A	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.
CHT4 - Clones (5)	.	T	.	.	.	.	A	.	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.
CHT4 - Clones (3)	.	T	.	.	.	.	A	.	T	.	.	.	.	.	T	.	.	.	.	.	A	.	.
CHT4 - Clones (2)	.	T	.	.	.	.	A	.	.	.	.	.	T	.	T	.	T	.	.	.	A	.	.
CHT4 - Clones (1)	.	T	C	.	.	.	A	.	T	.	.	.	.	.	T	.	.	.	.	.	A	.	.
CHT4 - Clones (1)	.	T	.	.	.	.	A	T	T	.	.	.	.	.	T	.	.	.	G	.	A	.	.
CHT4 - Clones (1)	.	T	.	.	.	.	A	.	.	T	.	.	.	.	T	.	.	.	.	.	A	.	.
CHT4 - Clones (1)	.	T	.	.	.	.	A	.	.	T	.	G	.	.	T	.	.	.	.	.	A	.	.
CHT6 - Direct 3rd	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
CHT6 - Clones (20)	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
CHT6 - Clones (1)	.	T	.	.	.	.	.	.	.	N	.	.	.	.	.	T	.	.	.	.	.	.	.
CHT6 - Clones (1)	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	.	.	.	.	.	.

Table 6 Direct sequencing and clone results for the fourth fragment of the human mtDNA HVRI (positions 16250-16382). Letters in red represent damage. The first clone sequence represents the consensus.

	16255	16258	16265	16266	16267	16290	16291	16293	16298	16301	16302	16308	16310	16311	16319	16322	16323	16325	16327	16336	16340	16345	16348	16349	16357	16361	16362
Reference	G	A	A	C	C	C	C	A	T	C	A	T	G	T	G	A	T	T	C	G	A	A	C	A	T	G	T
B001 - Direct 4th	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C
B001 - Clones (10)	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C
B001 - Clones (1)	.	.	.	.	.	T	.	.	.	.	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	.	C
B001 - Clones (1)	.	N	N	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C
B009 - Direct 4th	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B009 - Clones (11)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B009 - Clones (1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	C
B009 - Clones (1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	N	C
B009 - Clones (1)	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B023 - Direct 4th	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B023 - Clones (4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B023 - Clones (1)	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B023 - Clones (1)	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
B024 - Direct 4th	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B024 - Clones (15)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B026 - Direct 4th	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B026 - Clones (8)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B026 - Clones (1)	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	N	N	.	.	C

Table 6 cont'd

	16255	16258	16265	16266	16267	16290	16291	16293	16298	16301	16302	16308	16310	16311	16319	16322	16323	16325	16327	16336	16340	16345	16348	16349	16357	16361	16362
Reference	G	A	A	C	C	C	C	A	T	C	A	T	G	T	G	A	T	T	C	G	A	A	C	A	T	G	T
B038 - Direct 4th	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B038 - Clones (6)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B038 - Clones (2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	.	.	C
B038 - Clones (1)	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B038 - Clones (1)	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B038 - Clones (1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	.	.	.	.	.	.
B048 - Direct 4th	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B048 - Clones (11)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C
B064 - Direct 4th	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.
B064 - Clones (13)	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.
B064 - Clones (1)	N	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	C	.	.
B064 - Clones (1)	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.
B064 - Clones (1)	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.

Figure 1 Risk of reporting erroneous results when considering rate and distribution of damage

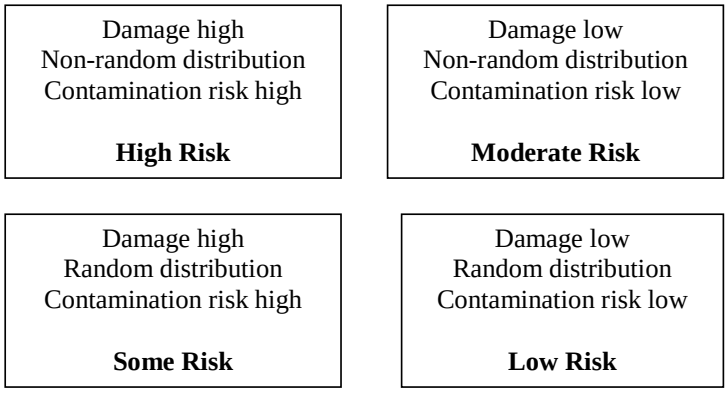


Figure 2 Total instances of damage induced by transitions and transversions among clones. Arrows indicate direction of damage and numbers correspond to total observed in the clones. **Figure 2A** Total hits of damage across all nucleotides among all clones. **Figure 2B** Total hits of damage across all nucleotides across unique clones

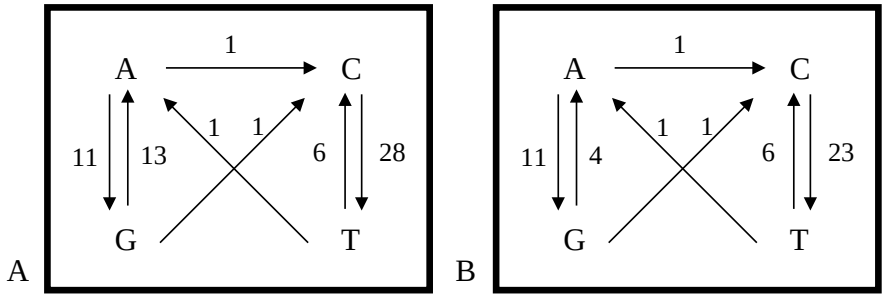


Figure 3 Total damage variation across all samples and clones (positions 16011-16382). Absolute damage was measured in number of hits per site across all clones sequenced at those positions. Peaks are labeled to show position numbers and provide comparison across the figures.

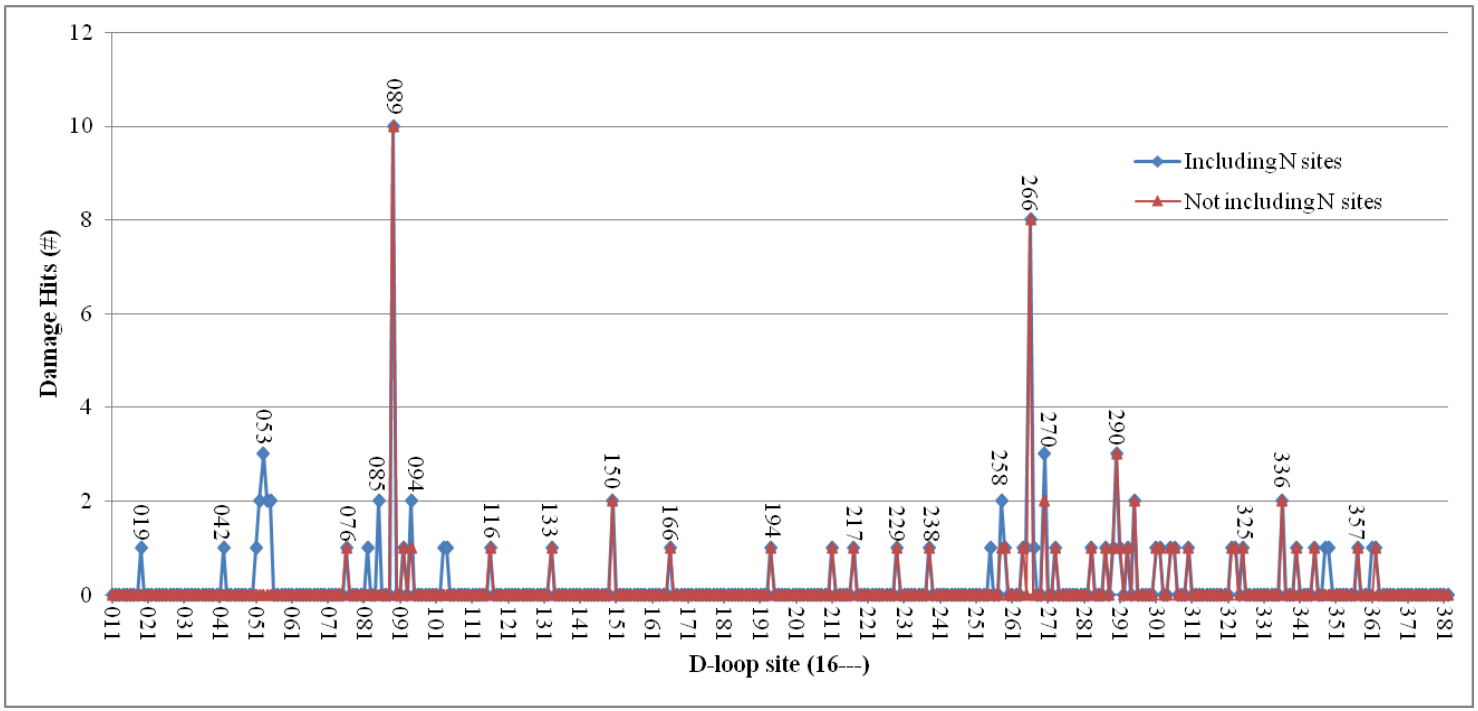


Figure 4 Unique damage variation across all samples and clones (positions 16011-16382). Unique damage was measured in number of hits per site across different clone haplotypes sequenced at those positions. Peaks are labeled to show position numbers and provide comparison across the figures.

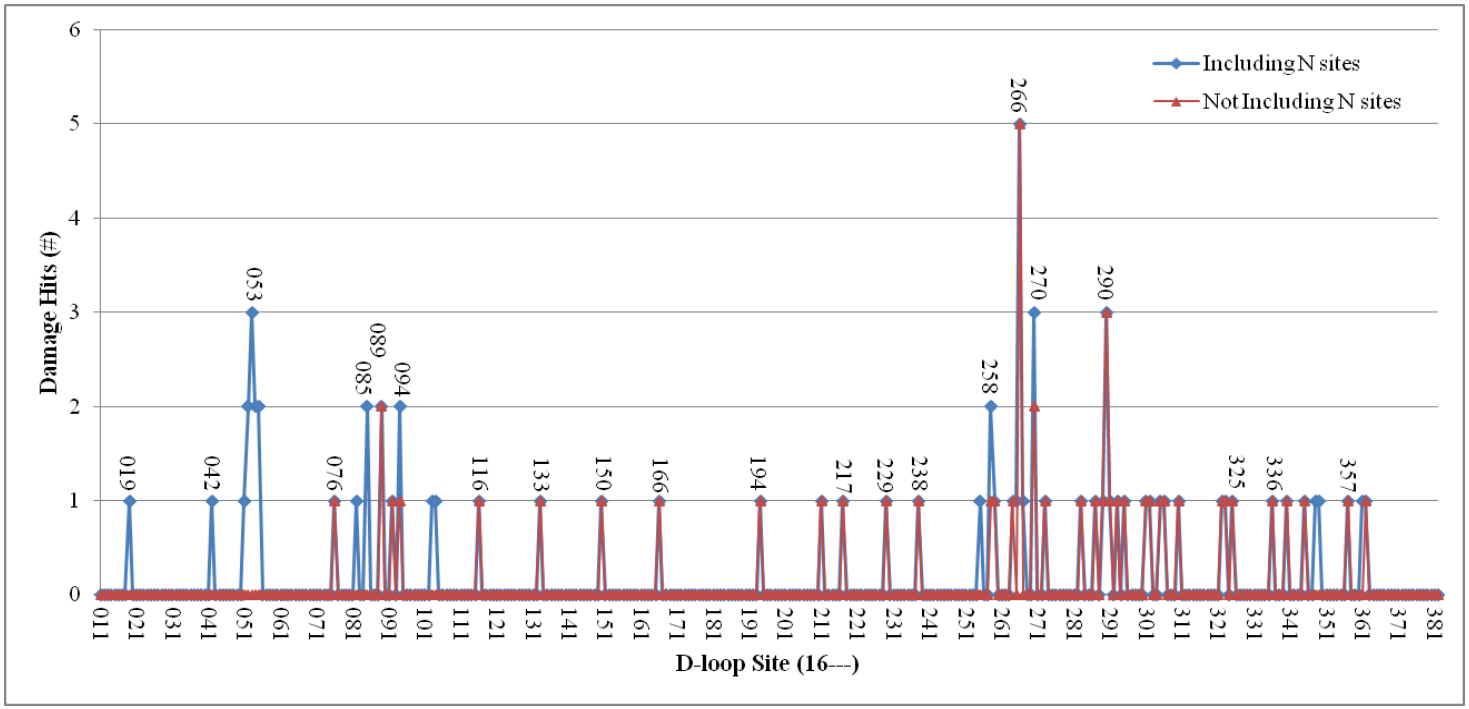


Figure 5 Relative rate of damage (x100) compared across total and unique damage. Rates were calculated as number of damage hits over the total number of clones (total damage), or the number of unique clone haplotypes (unique damage). Peaks are labeled to show position numbers and provide comparison across the figures.

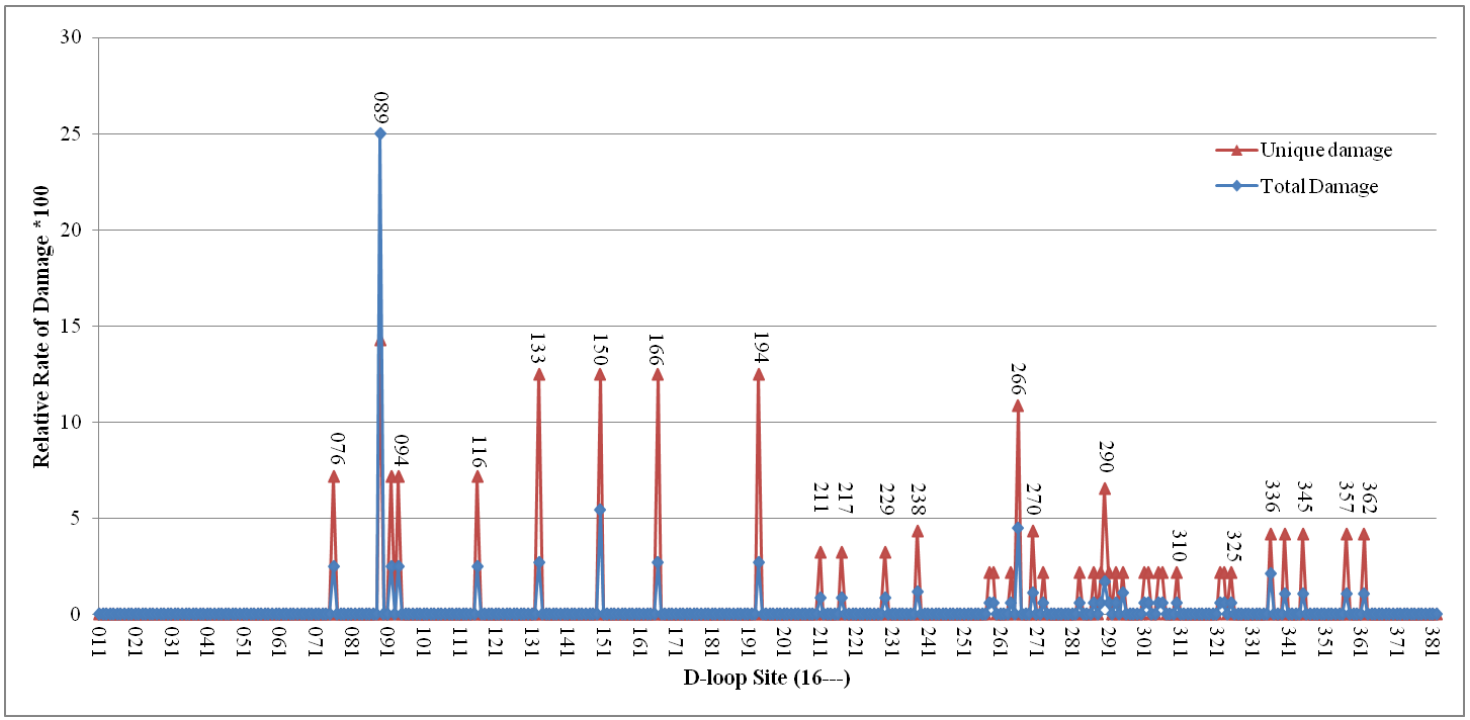


Figure 6 Observed and expected counts of total and unique damage rates (positions 16011-16382). Left: Total damage, Right: Unique damage.

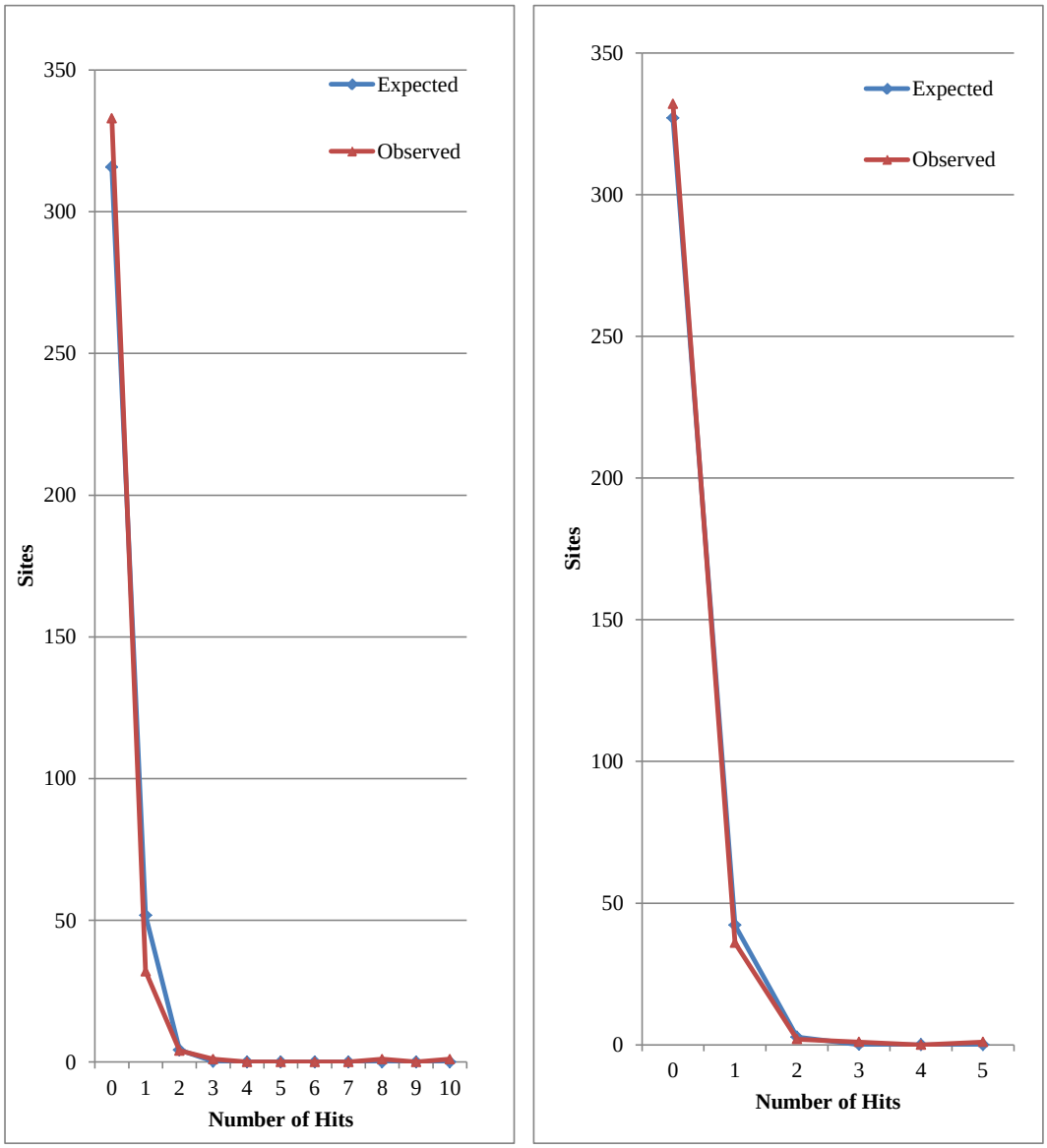
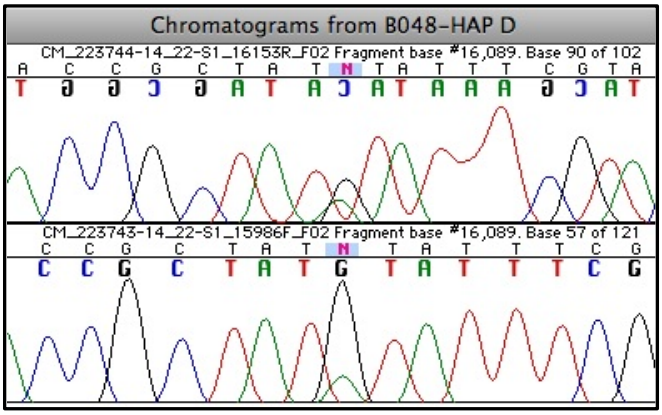


Figure 7 Chromatogram for site 16089 on sample B048 – D loop 1. Demonstrates competition of guanine and adenine within the chromatogram and classifies an N site.



DISSEMINATION OF RESEARCH FINDINGS

Publications

2011 Winters M, Barta JL, Monroe C, and Kemp BM "To clone or not to clone: Method analysis for retrieving consensus sequences in ancient DNA samples." *PLoS One* 6(6): e21247.

Presentations

2012 Barta JL, Monroe C, Teisberg J, Winters M, Flanigan K and **Kemp BM**. "One of the Key Characteristics of Ancient and Forensic DNA, Low Copy Number, May be a Product of its Extraction." Poster presented at the National Institute of Justice Conference, Arlington, VA.

2011 Monroe C, Barta JL, Kemp BM. "Overcoming PCR Inhibitors of Degraded DNA Using Various Thermostable Polymerases." Poster presented at the National Institute of Justice Conference, Arlington, VA.

2011 Barta JL, Monroe C, and **Kemp BM**. "Evaluating the Efficacy of Household Bleach in the Removal of Contamination from Bone Surfaces." Poster presented at the 80th Annual Meeting of the American Association of Physical Anthropologists. Minneapolis, MN.

2011 **Kemp BM**, Barta JL, Monroe C, Teisberg J, Runnells S, and Flanigan K. "One of the Key Characteristics of Ancient DNA, Low Copy Number, May be a Product of its Extraction." Poster presented at the 80th Annual Meeting of the American Association of Physical Anthropologists. Minneapolis, MN.

2011 Runnells S, Monroe C, Barta JL, and **Kemp BM**. "To clone or not to clone: Method analysis for retrieving consensus sequences in ancient DNA samples." Poster presented at the 80th Annual Meeting of the American Association of Physical Anthropologists. Minneapolis, MN.

2010 Barta JL, Monroe C and **Kemp BM**. "To bleach or not to bleach: Evaluating the efficacy of bleaching skeletal samples prior to DNA extraction" Presentation made at the 21st International Symposium on Human Identification, San Antonio, October 11-14. Special workshop: Problem samples.

2010 Barta JL, Monroe C and **Kemp BM**. "Evaluating the Efficacy of Bleach in the Removal of Contamination from Bone Surfaces" Poster presented at the National Institute of Justice Conference, Arlington, VA.

2009 **Kemp BM** and Monroe C. "NIJ Project to Enhance Methods for Studying Degraded DNA." Poster presented at the National Institute of Justice Conference, Arlington, VA