

Documenting ancient DNA quality via alpha satellite amplification and assessment of clone sequence diversity

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Abstract. C/G→T/A nucleotide alterations have been shown to hamper the straightforward interpretation of mitochondrial DNA sequence data derived from ancient tissues. Attempting to characterise this finding with respect to nuclear DNA, we contrasted two established protocols: (i) an enzymatic repair of damaged DNA, thereby translating and closing nicks in the DNA, and (ii) the application of *N*-phenacylthiazolium bromide, which cleaves glucose-derived protein crosslinks, presumably derived from Maillard reactions. We used medieval human bones that were refractory to standard PCR procedures. Due to negligible presence of short tandem repeat loci and also mitochondrial sequences, the extracted ancient DNA needed a higher copy PCR system to yield amplification products. The chosen PCR target was specific alphoid repetitive DNA with an experimentally determined minimum of 1000 copies per haploid genome. Alphoid repeat segments were generated from both contemporary DNA and DNA extracts of two human skeletons dating from 450-600 AD (omitting uracil *N*-glycosylase pre-treatment of the extracted samples), and were subsequently cloned and sequenced. The sequences were evaluated for the number and type of nucleotide alterations noted after the different pre-treatments, and were compared to our alphoid consensus sequence generated from modern DNA. Both methods failed to reflect the expected 32% variability among single alphoid repeats (accounting for locus-specific differences and polymerase errors) as well as to display the actual 2.88 ratio of transitions to transversions. Our data obtained from high-copy-number nuclear DNA mirror the phenomenon of sequence deviations observed in mitochondrial DNA extracted from old specimens.

Key words: alpha satellites, diagenetic alteration, DNA damage, DNA repair, human bones, PCR.

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Introduction

Numerous obstructions to DNA research become apparent when working with ancient tissues. Generally, a low efficiency of PCR using ancient DNA as template is observed. Given the putative presence of ancient DNA, a number of advances have been made, which may increase amplification efficiencies. Amongst them is the optimisation of the ancient DNA extraction procedure to increase both the quality and quantity of nucleic acids endogenous to the find (e.g. YANG et al. 1998), the removal of PCR inhibitors by purification steps (e.g. HÄNNI et al. 1995, SCHOLZ et al. 1998, KALMAR et al. 2000), the monitoring of contaminating DNA derived from microorganisms (e.g. PUSCH, SCHOLZ 1997) or from modern humans (e.g. PUSCH et al. 2000a), the use of specially adapted PCR protocols (e.g. PUSCH et al. 2000b) dealing with the shortcomings associated with the molecular status of old DNA, and the modification of ancient DNA itself prior to PCR-mediated amplification (FOO et al. 1992, POINAR et al. 1998, PUSCH et al. 1998). Whereas the first points mentioned have been addressed by straightforward approaches, the latter procedure offers a great scope for obtaining authentic genotypes, since it directly affects the native template, i.e. the isolated nucleic acids. In order to test the reliability (i.e. exclusion of contamination with biomolecules, reducing the number of frequently observed transitional nucleotide errors) and reproducibility (i.e. obtaining an identical genotype in independently performed experiments) of DNA data obtained from such modification procedures, we compared two protocols published previously (POINAR et al. 1998, PUSCH et al. 1998). This strategy was applied to ancient DNA samples where an amplification product could be reasonably expected, but from which no standard PCR products were obtained.

In the first assay series, we performed repair reactions (RRs) on extracted ancient DNA with the DNA polymerase I enzyme from *E. coli* and T4 DNA ligase. This approach focuses on the state of preservation of the chemically altered DNA consisting of nicked double strands due to hydrolysis, oxidation or enzymatic destruction. *E. coli* DNA polymerase I translates the nicks in the DNA and the remaining gaps are closed by the subsequent use of T4 DNA ligase. The second approach utilised *N*-phenacylthiazolium bromide (PTB, VASAN et al. 1996), which improves DNA retrieval from bones and coprolites by cleaving sugar-derived condensation products that otherwise may encapsulate nucleic acids (POINAR et al. 1998, HOFREITER et al. 2000, KRINGS et al. 2000). Specifically, advanced stages of the Maillard reaction (e.g. furan products) may result in extensive cross-linking of macromolecules (MAURON 1981, BUCALA et al. 1984, PAPOULIS et al. 1995), and have been proposed as components in ancient DNA extracts (POINAR et al. 1998, INNIS et al. 1990).

Here, we present the results of a comparative DNA improvement study employing two protocols published previously. High-copy-number alpha satellite re-

peats (e.g. MANUELIDIS 1978, SINGER 1982, MITCHELL et al. 1985, ALEIXANDRE et al. 1987, VISSEL, CHOO 1987, CHOO et al. 1991, YANG et al. 1998) were amplified from contemporary controls and ancient nucleic acids. Furthermore, we discuss aspects of the sensitivity, reproducibility, and reliability of ancient DNA manipulation protocols.

Materials and methods

Aseptic conditions

In order to prevent or minimise contamination with contemporary biomolecules when taking samples, the following precautions were taken. All working steps were carried out under sterile conditions, such as using clean benches, latex gloves, sterile working clothes, mouth masks and plexiglass face masks. All appliances and containers used for working with or storing the bone material were cleaned from any residues in several steps (stone meal, acetone, propan-2-ol) before and after use. In order to crosslink, i.e. to inhibit contaminating DNA fragments in PCR assays, the working areas were irradiated with UV-C light for approximately 2 hours. Decontamination of the containers and instruments used was carried out in two stages with substances capable of dissolving DNA and DNases (Molecular Bio-Products™ Inc.).

Sample processing

Samples were taken from the diaphysis region of the long extremity bones of 2 individuals (ind. 1, ind. 2) from the Neresheim burial ground (Germany) dating from 450-600 AD. The total sample consisted of 2 bone fragments of about 0.5 to 1.0 cm³ in order to make up for differences in DNA preservation due to variable storage conditions. The bone chips were removed with a hand mill (DBP Supra) by fitting it with a diamond separation disc (Ultraflex 912-EF). After the sample has been removed, a slice of about 1-2 mm was milled off the sides of each sample, cleaning it from spongiosa. For this purpose the mill was fitted with a new disc (6830 Orthodontros™). The bone samples were then mechanically ground into a fine powder with a vibration mill and sterile agate mortars. The bone powder was collected into sterile containers (Falcon BlueMax 2070) and stored at room temperature till further use.

Racemate contents

Approximately 1.0 mg of dried pulverised bone taken from the compacta was hydrolysed in 6 N DCl/D₂O. After evaporation to dryness, the sample was

derivatised to the N(O-)-TFA/ethyl esters, dissolved in toluene and analysed by selected-ion gas chromatography-mass spectrometry on a 2,6-dipentyl 6-butyryl γ cyclodextrin column. The ions m/z 140, 182 and 170, corresponding to alanine, leucine and aspartic acid, respectively, were selectively monitored (GERHARDT, NICHOLSON 1994).

DNA extraction

Substitution of collagenase for the commonly used proteinase K in standard protocols permits removal of collagen remnants from ancient DNA extracts, increasing the purity of DNA isolates (SCHOLZ, PUSCH 1997). After pulverisation of the samples, 0.1-0.5 g of bone powder was incubated at 25°C for 60 min with 0.3 U collagenase in 200 μ l of 1 \times PBS prior to lysis. If the chosen enzyme concentration of 0.3 U did not eliminate PCR inhibition, an aliquot of the remaining sample was further treated by applying another preparation cycle with an extended incubation time of 1.5h. Following enzymatic treatment, the ancient DNA isolates were purified three times with glass wool/Sephadex G-50 columns, then subjected to a phenol/chloroform/isoamylalcohol (25:24:1) extraction, and finally precipitated with 2.5 vol. EtOH_{abs}, and 1/10 vol. 3 M NaOAc (pH 5.3). The pellet was washed twice with 300 μ l 70% EtOH, air dried, and subsequently dissolved in 10-15 μ l of TE buffer (pH 8.0) prior to DNA manipulation via RR, which was essentially performed as described (PUSCH et al. 1998). For the assay in which PTB (VASAN et al. 1996) was used, we followed the protocols for DNA extraction as described by POINAR et al. (1998).

Amplification of alphoid repetitive DNA

A PCR protocol was accomplished with primers locating to a 2,712 bp DNA centromeric segment (Acc. No. M13882). Primer sequences (given in Figure 2), PCR set-up, and protocols for thermal cycling for amplifying a 211-bp PCR product out of M13882 were essentially taken from YANG et al. (1998). Distribution of α -amplicons and allele-specific amplification patterns were identified using a whole genome rodent/human radiation-reduced hybrid panel (Coriell Cell Repositories, USA). The PCR products were separated on 2.0% agarose gels and assessed after ethidium bromide staining and subsequent visualisation of the DNA fragments on a UV transilluminator.

In addition, contemporary human DNA was isolated from peripheral blood of two recent individuals (MILLER et al. 1988), and served as template (10 ng) in control amplifications (control A and B).

Cloning of PCR products and DNA sequencing

The remaining aliquot out of positive PCR assays was purified with Centricon one-way concentrator vials (Amicon). Ligation reactions were performed in a vol-

ume of 8 μ l using a protocol for temperature-cycle ligation (PUSCH et al. 1997). A total of 15 ng DNA and a molecular ratio of 1:4 of pCR2.1 (Invitrogen) plasmid vector to insert was employed. Chemical transformation of recombinants into *E. coli* bacterial cells was done as recommended by the manufacturer (Invitrogen). Transfected cells were grown in 450 μ l SOC medium for 50 min before plating on selective ampicillin-kanamycin-IPTG-Xgal luria broth agar plates. Recombinant white clones were tested for insert integrity in colony PCRs applying M13 forward and reverse primers (MWG Biotech). The temperature profile was carried out with 30 cycles at 92°C for 20 sec, 55°C for 15 sec, and 72°C for 20 sec, and was finished by a final extension at 72°C for 5 min. Size determination of the plasmid inserts was done by agarose gel electrophoresis. DNA was released from the respective recombinants by using a minilysate method (ZHOU et al. 1990) followed by two purifications with PEG 5000. Cloned PCR products were sequenced with BigDye cycle-sequencing chemistry (ABI Perkin Elmer) according to the manufacturer's instruction. One third of the purified mixes out of the cycle-sequencing reactions were loaded onto 6% denaturing polyacrylamide gels and analysed on an automated DNA sequencer (ABI). Analysis of DNA sequence electropherograms was performed with ABI Sequence Analysis 3.4 software and the Lasergene package for DNA sequence alignments (SeqMan version II). In multialignments a "hot spot" was defined as alteration in vertical alignment position with a frequency of ≥ 3 .

Results

For this study we used non-inhibitory DNA extractions from 2 human skeletons dating from 450-600 AD. We determined in initial test series that they were refractory to PCR-mediated amplification of single copy loci and also multicopy mitochondrial segments when standard protocols were applied. However, the state of amino acid preservation in the samples was compatible with DNA preservation, and indicator contents (i.e. ratio of D to L enantiomers) of the amino acid aspartic acid ($\ll 0.08$) indicated that amplifiable DNA should be present in the samples (POINAR et al. 1996, COOPER et al. 1997). Further, D/L values of leucine and alanine were smaller than D/L aspartic acid, ruling out gross contamination of the samples (POINAR et al. 1996).

In order to achieve amplification products from these samples we utilised a high-copy-number PCR system that amplifies alphoid DNA localised at centromeres of mammalian chromosomes. Due to its tandem repetitive nature, the available template copy number is expected to be superior to that of multi-copy PCR systems employing mitochondrial DNA.

To calculate the available PCR template copy number of alphoid repeats, the spectrum and number of chromosomal templates in modern DNA was deter-

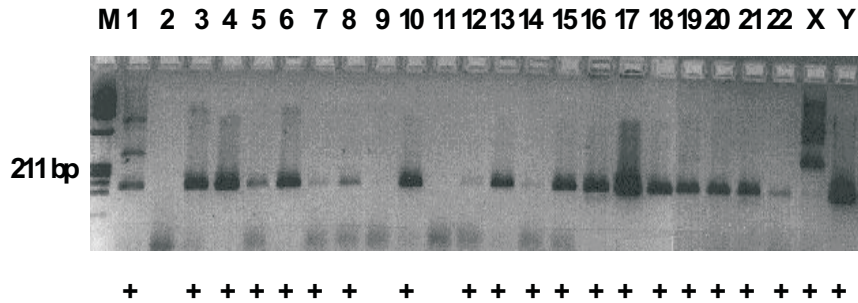


Figure 1. Distribution of an alpha satellite sequence tag using a whole genome radiation-reduced cell hybrid panel. Lanes 1-22 correspond mainly to the respective human autosome (i.e. #1-#22, top), whereas lanes 23 and 24 refer to amplification patterns obtained from the DNAs of human sex chromosomes (X and Y respectively). M is a symbol for “DNA marker” and “+” denotes positive PCRs (bottom). Note that alphoid PCR results in increased sensitivity but not specificity of the test. Amplifications using rodent and human nucleic acids as the templates, however, are not directly comparable since a ten to hundred-fold excess of rodent DNA produces signals at all. Thus, PCR fails in the presence of only 5 ng of DNA of Chinese hamster or mice, while the corresponding segments in humans (probably due to higher copy numbers) are exclusively amplified.

mined with the use of a whole genome hybrid panel. Homologous centromeric regions matching the primer pair sequences were amplified in almost all human chromosomes, except for chromosomes 2, 9, and 11 (Figure 1).

Assuming an effective copy number of 50 templates for the initiation of PCR (KRINGS et al. 1997), and assuming further that only 50 copies are available from each of the 21 chromosomes (that had been determined positive for this sequence tag) an average minimum of 1,050 sites per haploid genome can be assessed.

Consistent with the previous PCR failures, it was impossible to amplify alpha satellites from the two ancient samples applying standard PCR protocols, but we obtained the expected, specific 211 bp product from the two modern reference samples. Sequencing of the cloned PCR products confirmed the base order for alpha satellite repeats matching from position 75-285 with the 2,712 bp database reference sequence (Acc. No. M13882). The overall similarity of this specific segment with the alpha consensus was tested with the RepeatMasker algorithm. Transitions as well as transversions locating to the primer regions in M13882 are responsible for this PCR system failing to randomly amplify all of the alpha satellites present in the genome (Figure 2). The sequence deviations at positions 123 and 189 are explained by deviating M13882 sequences, as originally deposited in the database, and were disregarded in further analyses (Figure 2). BlastN homology searches identified another alphoid segment as being closely related (Acc. No. X06646). In subsequent multialignment procedures these sequences were used as master references (Figure 3).

modern.Seq	1	<u>CAAATCCCGAGTTGAACTT</u> TCCTTTCAA-AGTTCACGTTTGAAACACTC	49
		v v v ? ii - vv vv	
ALR/alpha#Sat	34	CAACTCACAGAGTTGAACMTTCCTTTTGATAGAGCAGTTTGAAACACTC	83
modern.Seq	50	TTTTTGCAGGATCTACAAGTGGATATTTGGACCACTCTGTGTCCTTCGTT	99
		i i i ? ? i v v? ?	
ALR/alpha#Sat	84	TTTTTGTAGAATCTGCAAGTGGATATTTGGASCKCTTTGAGMCTTCGKT	133
error		<i>C(123)</i>	
modern.Seq	100	CGAAACGGGTATATCTTCACATGACATCTAGACAGAAGCATCCTCAGAAG	149
		v v i v v i i	
ALR/alpha#Sat	134	GGAAACGGGAATATCTTCACATAAAAACTAGACAGAAGCATCTCAGAAA	183
error		<i>T(189)</i>	
modern.Seq	150	CTTCTCTGTGATGACTGCATTCAACTCACGGAGTTGAACACTCC <u>TTTTGA</u>	199
		i vi i ?i	
ALR/alpha#Sat	184	CTTCTTTGTGATGTTTGCATTCAACTCACAGAGTTGAACMTTCCTTTTGA	233
modern.Seq	200	<u>GAGCGAGTTTT</u>	211
		v v	
ALR/alpha#Sat	234	TAGAGCAGTTTT	245

Figure 2. Comparison of a 211-bp alphoid DNA sequence termed “modern.Seq” against the ALR/alpha#Satellite/centromeric consensus (position 34 to 245) using RepeatMasker software. Score obtained was SW 1246, with percentage diversity of 16.51, percentage deletions of 0.47, and no insertions (0.00). Ratio of transitions/transversions: 0.81. Primer sites used for deducing oligonucleotide sequences are bold faced and underlined. Sequencing errors in M13882 are italicised at positions 123 and 189 of the deposited reference sequence. Abbreviations used are “i” for transition and “v” for transversion.

Both ancient DNA improvement protocols, the enzymatic repair reaction (RR) and the PTB-induced DNA release, were applied to samples of the two ancient skeletons from Neresheim, Germany. Alphoid repeats were subsequently amplified from the samples with the use of identical conditions for thermal cycling and PCR set-up resulting in PCR fragments of the expected size. In parallel, no PCR products were obtained for mitochondrial HVRI segments and the less abundant single-copy loci. The alpha satellite amplicons were subcloned into plasmid vectors and a total of 171 inserts were sequenced with universal primers. In addition, DNA of the two modern controls (A and B) was isolated in a straightforward way, then amplified and the amplicons were subcloned and sequenced. Here, a total of 89 sequences was determined to use them as a reference in comparison to the sequences originating from the treated ancient DNA samples (Table 1). We first investigated conformity or discordance in the pool of modern sequences, and subsequently compared the variability of the contemporary sequences with

the variability found in DNA following the “improvement” protocols. It is expected that an optimal ancient DNA protocol leads to PCR products displaying a comparable degree of variability/identity as determined for the modern intact nucleic acids (amplified without previous optimisation). Otherwise, a significant mismatch needs to be explained by, e.g., incorporation of wrong bases into a nicked DNA strand using RR, or increased *Taq* polymerase errors in the early cycles by an inefficient PTB treatment. The obtained results and the mathematical evaluation of the assays are summarised in Tables 1 and 2.

Table 1. Characterisation of alpha satellite sequences amplified from modern (control) and ancient DNA

Method and assay no.	Total seq.	Altered seq.	No. of alterations	Transition (i)	Transversion (v)	Ratio i : v
Control A	41	18	30	22	8	2.75
Control B	48	10	12	9	3	3.00
RR ind. 1	46	21	36	24	12	2.00
RR ind. 2	41	17	27	19	8	2.38
PTB ind. 1	46	26	29	15	14	1.07
PTB ind. 2	38	17	20	18	2	9.00

RR = samples subjected to repair reaction; PTB = samples treated with *N*-phenacylthiazolium bromide.

Table 2. Determination of sequence variability in modern (control) and ancient DNA

Method	var1 [%]	var2 [%]	var1/var2	Mean ratio I : v
Controls	32.36	49.09	0.66	2.88
RR	43.46	72.06	0.60	2.19
PTB	50.63	59.84	0.85	5.04

var1 = altered sequences per total number of sequences; var2 = alterations per total number of sequences
RR = samples subjected to repair reaction; PTB = samples treated with *N*-phenacylthiazolium bromide.

Analysing the pool of modern sequences, we determined a mean variability at 32.36% (i.e. altered sequences per total number of sequences, var1, Table 2). However, both optimisation protocols applied to the ancient DNA failed in reaching such low values for sequence variability. An increased mean variability can be seen in both protocols (RR: 43.46% and PTB: 50.63%). The different results can be reliably monitored by the ratio of transitions versus transversions. In the modern DNA we determined a mean value of 2.88, which is in good agreement with values obtained by others (COLLINS, JUKES 1994). Conformity between both amplification experiments A (2.75) and B (3.00) (Table 1) emphasised that the chosen PCR system generates reproducible data. Whereas the RR approach underestimates this value in both independent PCRs (mean: 2.19), the PTB

Table 3. Nucleotide positions and nucleotide alterations of "mutational" hot spots in modern (control) and ancient DNA

Method, assay no.	No. of hot spots	Nucleotide position	Mutational event	Nucleotide alteration
Control A	2	106, 141	i, i	G > A, C > T
Control B	1	141	i	C > T
RR ind. 1	3	45, 128, 141,	v, i, i	C > A, T > C, C > T
RR ind. 2	1	141	i	C > T
PTB ind. 1	4	45, 63, 88, 141	v, v, v, i	C > A, T > G, T > G, C > T
PTB ind. 2	1	141	i	C > T

i = transition; v = transversion; RR = samples subjected to repair reaction; PTB = samples treated with *N*-phenacylthiazolium bromide.

approach reaches a value of 5.04 (Table 2), thereby exceeding the estimates of the other assays by a factor of ~2. Moreover, the results of the two PCRs using the Neresheim extracts are quite similar in the RR approach (2.00 for ind. 1 and 2.38 for ind. 2), but differ by a factor of ~9 between the two PTB assays (1.07 for ind 1 and 9.00 for ind. 2) (Table 1). Further, the examination of "mutational" hot spots within a DNA sequence is a good criterion for the impact of an improvement protocol. Hot spots in the pool of modern sequences are the more common transitions at positions 106 (G > A) and 141 (C > T) in M13882 (Table 3). Differently, an accumulation of further-thus artificially created-alterations occurs in the pool of ancient sequences derived from Neresheim ind. 1. Amongst the known sites at nucleotide 106 and 141 from the modern DNA amplifications, mainly transversions are found, regardless of which protocol was used for DNA optimisation (Figure 3, Table 3). Since non-corrected "hot spots" may erroneously be featured as authentic genotypes, the interpretation of the base order of old nucleic acids must be considered with caution.

Discussion

The development of novel techniques in palaeogenetic research appears to be a promising step in the authentication procedure. So far, no protocol established for this purpose can be called optimal. Recent improvements have mostly been aimed at establishing variations of the PCR technology itself to achieve a higher methodological specificity. However, an improvement of the yield (i.e. increase in methodological sensitivity) as well as of quality of ancient DNA present in a find appears necessary. To this end, we investigated two methods that

have been recently established to manipulate DNA during DNA extraction and prior to PCR application. The goal of such manipulation is to obtain PCR results with maximum specificity, and to amplify templates that have been refractory to PCR-mediated multiplication without optimisation strategies.

The sequence that is obtained should be one with the correct, i.e. authentic base order. Reproducibility of results between extracts and PCRs does not guarantee the correct sequence, but can also be explained by the generation of reproducible amplification artifact(s) (HANSEN et al. 2001, HOFREITER et al. 2001). In our study we observed this phenomenon, where mutational hot spots were artificially created when DNA of Neresheim ind. 1 treated for improvement was used for amplification. It remains to be clarified why more transversions than transitions are seen in (PTB-treated) ind. 1 sequences, given the fact that transitions have been determined to be the more frequent event (HANSEN et al. 2001). The generation of non-authentic genotypes is a serious problem in palaeogenetics when it relies on sequence comparison between a fossil or extinct specimen and a closely related species. To reliably construct a reproducible base order of the desired ancient DNA segment, it remains vital to investigate several PCR products derived from different PCR approaches for each individual (PUSCH et al. 1998). Unfortunately, after testing two protocols that were proposed for DNA improvement (the first one enzymatically repairing damaged DNA by translating and closing nicks in DNA, the second one utilising *N*-phenacylthiazolium bromide to cleave glucose-derived protein crosslinks), ancient DNA refractory to standard PCR showed a significant deviation in base sequence, as compared to controls. Application of *E. coli* uracil *N*-glycosylase, which has been recently described as a tool for obtaining the correct base order from ancient nucleic acids (HOFREITER et al. 2001), did not improve the ancient material in our studies (unpubl. observation). Additional techniques to overcome this severe shortcoming have not been developed as yet, frequently the number of artificially generated errors will increase with enhanced PCR sensitivity. Failure to differentiate between mutations and artifacts prevents a decision for authentic or erroneous base order, and thus forecloses scientific evaluation of ancient DNA. Improved understanding of the mechanisms causing such phenomena may hopefully lead to mathematical models that will help to interpret these situations. It is expected that such mechanisms are closely connected to the still poorly understood diagenetic alterations occurring in bone chemistry. Since the study presented here pertains only to two skeletons with a very similar origin, the results are, however, very preliminary. Thus, the goal of future studies will be a detailed examination of bone specimens offering different diagenetic characteristics, thereby extending the approach to a larger number of samples with different degrees of DNA damage.

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