

Assessing the Fidelity of Ancient DNA Sequences Amplified From Nuclear Genes

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Manuscript received August 17, 2005
Accepted for publication October 25, 2005

ABSTRACT

To date, the field of ancient DNA has relied almost exclusively on mitochondrial DNA (mtDNA) sequences. However, a number of recent studies have reported the successful recovery of ancient nuclear DNA (nuDNA) sequences, thereby allowing the characterization of genetic loci directly involved in phenotypic traits of extinct taxa. It is well documented that postmortem damage in ancient mtDNA can lead to the generation of artifactual sequences. However, as yet no one has thoroughly investigated the damage spectrum in ancient nuDNA. By comparing clone sequences from 23 fossil specimens, recovered from environments ranging from permafrost to desert, we demonstrate the presence of miscoding lesion damage in both the mtDNA and nuDNA, resulting in insertion of erroneous bases during amplification. Interestingly, no significant differences in the frequency of miscoding lesion damage are recorded between mtDNA and nuDNA despite great differences in cellular copy numbers. For both mtDNA and nuDNA, we find significant positive correlations between total sequence heterogeneity and the rates of type 1 transitions (adenine → guanine and thymine → cytosine) and type 2 transitions (cytosine → thymine and guanine → adenine), respectively. Type 2 transitions are by far the most dominant and increase relative to those of type 1 with damage load. The results suggest that the deamination of cytosine (and 5-methyl cytosine) to uracil (and thymine) is the main cause of miscoding lesions in both ancient mtDNA and nuDNA sequences. We argue that the problems presented by postmortem damage, as well as problems with contamination from exogenous sources of conserved nuclear genes, allelic variation, and the reliance on single nucleotide polymorphisms, call for great caution in studies relying on ancient nuDNA sequences.

WHEN an organism dies, its DNA starts degrading at a rate that is believed to be highly dependent on the environment and subsequent conditions of storage (PÄÄBO 1989; LINDAHL 1993; HÖSS *et al.* 1996; POINAR *et al.* 1996; HOFREITER *et al.* 2001a; SMITH *et al.* 2001; PÄÄBO *et al.* 2004; WILLERSLEV *et al.* 2004a; WILLERSLEV and COOPER 2005). Postmortem DNA is subject to degradation by microorganisms, soil invertebrates, and cellular nucleases, in addition to modifications by spontaneous chemical reactions such as hydrolysis and oxidation (LINDAHL 1993; HOFREITER *et al.* 2001a; GILBERT and HANSEN 2006). While some

types of postmortem DNA modification block the extension of polymerase enzymes, thus rendering the molecules unsuitable as template for PCR, others, termed miscoding lesions, allow for amplification, but result in the incorporation of erroneous bases during PCR (PÄÄBO 1989; LINDAHL 1993; HÖSS *et al.* 1996; POINAR *et al.* 1998; HANSEN *et al.* 2001; HOFREITER *et al.* 2001b; GILBERT *et al.* 2003a,b; BANERJEE and BROWN 2004; WILLERSLEV *et al.* 2004b; MITCHELL *et al.* 2005; GILBERT and HANSEN 2006). The most commonly observed miscoding lesions in ancient DNA (aDNA) studies are the transitions adenine → guanine (A → G), cytosine → thymine (C → T), G → A, and T → C (HANSEN *et al.* 2001; HOFREITER *et al.* 2001b; GILBERT *et al.* 2003b). Although four different transitions have been observed, it has been argued that it is possible to differentiate the transitions into only two complementary groups, termed type 1 (TS1: A → G/T → C) and type 2 (TS2: C → T/G → A) transitions, caused putatively by the

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. DQ318533–DQ318562.

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deamination of adenine to hypoxanthine (A → H) and the deamination of cytosine (and its homolog 5-methyl cytosine) to uracil (and thymine), respectively (HANSEN *et al.* 2001; HOFREITER *et al.* 2001b; GILBERT *et al.* 2003b). For example, consider a single cytosine to uracil (C → U) deamination event on the light strand of mitochondrial DNA (mtDNA). The C → U event will be observed as a C → T transition on any light strand sequences amplified directly from the original damaged template, but any of the derived complementary heavy strand sequences will exhibit a G → A transition. Similarly, any sequences derived directly from the amplification of an A → H event will be exhibited as either an A → G or T → C transition. Intriguingly, even though miscoding lesions generating both type 1 and type 2 transitions are recorded for DNA in solution (deamination of cytosine with a rate ~30–50 times higher than that for adenine; LINDAHL 1993), it is still debated whether the deamination of adenine actually plays a role in the generation of type 1 events in aDNA or whether they are simply due to regular DNA polymerase errors (HOFREITER *et al.* 2001b; GILBERT *et al.* 2003b; PÄÄBO *et al.* 2004).

Although mtDNA is most frequently used in aDNA research, a number of recent studies have reported the successful retrieval of low- and single-copy nuclear DNA (nuDNA) sequences (GREENWOOD *et al.* 1999, 2001; BUNCE *et al.* 2003; HUYNEN *et al.* 2003; JAENICKE-DESPRES *et al.* 2003; ORLANDO *et al.* 2003; POINAR *et al.* 2003; NOONAN *et al.* 2005). Such studies represent important breakthroughs, as nuDNA can be used to answer previously intractable questions, such as the establishment of phenotypic characteristics (BUNCE *et al.* 2003; JAENICKE-DESPRES *et al.* 2003) and the resolution of deep phylogenetic splits (POINAR *et al.* 2003). However, nuclear genes are typically 5000–10,000 times less abundant per cell than those of mitochondrial origin, which is probably the reason for ancient nuDNA being much more difficult to amplify than mtDNA from the same aDNA extracts (POINAR *et al.* 2003).

To date, studies that characterize DNA damage in fossil remains have been restricted to mtDNA (HÖSS *et al.* 1996; HANSEN *et al.* 2001; HOFREITER *et al.* 2001a; GILBERT *et al.* 2003a,b; THREADGOLD and BROWN 2003; BANERJEE and BROWN 2004; GILBERT *et al.* 2005). Although one study has reported substitutions in PCR-amplified ancient nuDNA sequences, the authors attribute the observed changes to mutagenic effects introduced under PCR reactions (PUSCH *et al.* 2004), although this remains questionable (SERRE *et al.* 2004a). Despite the lack of publications, the characterization of damage in ancient nuDNA remains important to the field. In particular, the fact that miscoding lesions can result in modification of the consensus sequence amplified from ancient mtDNA (HANDT *et al.* 1996; GILBERT *et al.* 2003a; BANERJEE and BROWN 2004; HEBSGAARD *et al.* 2005), along with the overall low

abundance of nuDNA templates relative to mtDNA templates, carries the implication that sequences amplified from ancient nuDNA are at increased risk of containing sequence errors. Furthermore, nuDNA sequences generated from fossil remains often are single nucleotide polymorphisms (SNPs), and the determination of SNPs as real or as the result of postmortem damage is crucial.

In this study we investigate the frequency and types of miscoding lesions in various mtDNA and nuDNA markers across a variety of fossil specimens of different ages preserved under different environmental conditions. In addition, we discuss some of the factors that need to be considered when amplifying, sequencing, and interpreting nuclear data from archival and fossil remains.

MATERIALS AND METHODS

We analyzed previously unreported DNA sequences extracted from permafrost-preserved bones of the woolly rhinoceros (*Coelodonta antiquitatis*, $n = 2$) and the lion (*Panthera leo spelaea*, $n = 6$), as well as temperate-preserved bones of the pig (*Sus scrofa*, $n = 5$) and the female moa (*Dinornis robustus*, $n = 4$). Not included in the comparative study were amplification results from one woolly rhinoceros, two pigs, and 28 lion specimens yielding mtDNA sequences but no nuDNA sequences and from three woolly rhinoceroses and 88 lion specimens yielding neither mtDNA nor nuDNA sequences. Additionally, published mtDNA and nuDNA clone sequences from desert-preserved ground sloth coprolites and permafrost-preserved woolly mammoth and woolly rhinoceros bones and teeth ($n = 6$) were included in the analyses (POINAR *et al.* 1996, 2003; GREENWOOD *et al.* 1999, 2001; ORLANDO *et al.* 2003). Published sequences from ancient human remains were not included due to the high risk of contamination in such studies (COOPER and POINAR 2001; HOFREITER *et al.* 2001b; PÄÄBO *et al.* 2004; WILLERSLEV and COOPER 2005). In total the data set comprised mtDNA and nuDNA sequences from 23 specimens (for sample details, see Table 1 and supplemental material at <http://www.genetics.org/supplemental/>).

Bone samples were collected, DNA extracted, and PCR amplified following established aDNA protocols: Using a Dremel tool, ~1-cm³ fragments ~0.5 cm in depth were removed from the bones. A Braun Mikrodismembrator was used to grind samples. Grinding equipment (stainless-steel balls and cups, rubber washers) was thoroughly bleached between each use. Decalcification was done in 5–30 vol of 0.5 M EDTA (pH 8) overnight at room temperature. The sediment was collected by centrifugation and digested with 0.25 mg/ml proteinase K/8 mg/ml DTT overnight at 50°–55°. The samples were extracted twice with phenol and once with chloroform, and the DNA was recovered and up-concentrated with Centricon-30 (Amicon, Beverly, MA) devices. The proofreading polymerase Platinum Taq Hifidelity (Invitrogen, San Diego) was used in PCR amplification to minimize the generation of DNA polymerase errors that can mimic errors caused by miscoding lesions (HANSEN *et al.* 2001). PCR amplifications were performed in 25- μ l volumes adding 0.02- to 1- μ l DNA extracts, 0–2 mg/ml bovine serum albumin (BSA), 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl (pH 8.3), 0.8 mM dNTPs, 1 mM of each primer, and 1 unit of DNA polymerase. Thermal cycling conditions were typically 40 cycles of 95°/52°–66°/68° (30–90 sec each) for mtDNA amplifications and 40–50 cycles of 95°/52°–55°/68° (45–60 sec each) for nuDNA amplifications. BSA was added to

TABLE 1
Sample details

Sample name ^a	Age ^b (YBP)	Specimen type ^c	Environment ^d	Species ^e	Sequence data ^f		Sequence region ^g	
					mtDNA	NuDNA	mtDNA	NuDNA
Mp-Wrangel	4590 ± 50	Bone	Permafrost	<i>Mammuthus primigenius</i>	4/556	14/1596	cyt b	Micro-satellites
Mp-Alaska8460	13,775 ± 145	Tooth	Permafrost	<i>Mammuthus primigenius</i>	14/2098 ⁱ	131/7252	cyt b	vWf,a2ab,irib, numt, micro-satellite
Mp-Siberia	26,000 ± 1600	Bone	Permafrost	<i>Mammuthus primigenius</i>	4/556	10/1140	cyt b	Micro-satellites
SC7400	60–70,000 ^h	Tooth	Temperate cave	<i>C. antiquitatis</i>	43/7463 ⁱ	4/680	cyt b	Numt
SC81205	40–45,000 ^h	Tooth	Temperate cave	<i>C. antiquitatis</i>	10/1820 ⁱ	9/1260	12S	Numt
PIN 3342-103	>49000	Bone	Permafrost	<i>C. antiquitatis</i>	47/11697 ⁱ	12/3552	12S, cyt b ^j	Numt
PIN 3100-169	43700 ± 1000	Bone	Permafrost	<i>C. antiquitatis</i>	6/3842	3/837	12S ^j	Numt
Ua11835	19875 ± 215	Cropolite	Varm cave	<i>Nothrotheriops shastensis</i>	76/6671	115/6813	12S	28S,vW, Crem, PCLB4
RB91	12,450 ± 60	Bone	Permafrost	<i>P. leospelaea</i>	10/1440	5/720	atp8	Numt
RB75	12,090 ± 80	Bone	Permafrost	<i>P. leospelaea</i>	16/2304	2/288	atp8	Numt
RB42	>50,600	Bone	Permafrost	<i>P. leospelaea</i>	9/1296	9/1296	atp8	Numt
RB44	54,100 ± 1800	Bone	Permafrost	<i>P. leospelaea</i>	8/1152	24/3456	atp8	Numt
RB41	46,200 ± 1500	Bone	Permafrost	<i>P. leospelaea</i>	9/1296	3/432	atp8	Numt
RB46	>61,500	Bone	Permafrost	<i>P. leospelaea</i>	7/1008	14/2016	atp8	Numt
GL92	91	Bone	Museum	<i>S. scrofa</i>	8/1712	6/1038	D-loop	CD45
GL71	86	Bone	Museum	<i>S. scrofa</i>	7/1493	8/1384	D-loop	CD45
GL55	46	Bone	Museum	<i>S. scrofa</i>	6/1284 ⁱ	6/1038	D-loop	CD45
GL45	98	Bone	Museum	<i>S. scrofa</i>	6/1284	7/1211	D-loop	CD45
GL76	50	Bone	Museum	<i>S. scrofa</i>	8/1712	8/1384	D-loop	CD45
Moa716	700–5000 ^h	Bone	Temperate cave	<i>D. robustus</i>	15/3031	15/615	D-loop	Kw1, ^j adh
Moa237	700–5000	Bone	Temperate cave	<i>D. robustus</i>	15/3031	16/656	D-loop	Kw1, adh
Moa660	613 ± 90	Bone	Temperate cave	<i>D. robustus</i>	14/2828	15/615 ⁱ	D-loop	Kw1, adh
Moa799	1–3000 ^h	Bone	Dry swamp	<i>D. robustus</i>	16/3232	32/2138	D-loop	Kw1, ^j adh

YBP, years before present.

^a Data sources: Moa716, Moa237, Moa660, and Moa799 are from BUNCE *et al.* (2003) and data generated in this study; Mp-Wrangel, Mp-Siberia, and MP-Alaska8460 are from GREENWOOD *et al.* (1999, 2001) and data generated in this study; Ua11835 is from POINAR *et al.* (1998, 2003); SC81205 and SC7400 are from ORLANDO *et al.* (2003); GL92, GL71, GL55, GL45, and GL76 are from LARSON *et al.* (2005) and this study; RB41, RB42, RB44, RB46, RB75, and RB91 are from this study; PIN3342-103 and PIN3100-169 are also from this study.

^b Age of specimen based on museum records, accelerator mass spectrometer dating, or on stratigraphy.

^c Type of specimen.

^d Type of original preservation environment.

^e Species name of specimen.

^f Sampling effort. The numbers of mitochondrial or nuclear clones/the total length of the sequences in base pairs. The average number of clones is very similar for mtDNA (16; SD 17) and nuDNA (20; SD 33).

^g Name of the mitochondrial and nuclear sequences studied.

^h Age of specimen based on stratigraphy.

ⁱ Results were independently replicated.

^j Results were quantified by real time PCR.

the PCR to overcome inhibition. Additionally, dilutions (1:10–1:50) of the DNA extracts were attempted. For primer details and amplification conditions, see Table 2. The amplification products were cloned and sequenced following established procedures (WILLERSLEV *et al.* 1999).

Strict protocols were followed to minimize the risk of sample and extract contamination with exogenous sources of DNA, including the use of aDNA facilities (physically isolated from the laboratories where postamplification manipulation is performed), the incorporation of extraction and PCR blank controls at ratios of 1:5 and 1:1–2, respectively, and quantification of a few of the extracts by real time PCR using SYBR green detection chemistry (Applied Biosystems, Foster

City, CA) (for details see supplemental material at <http://www.genetics.org/supplemental/>). Independent replication was carried out for a subset of results in dedicated aDNA facilities in Copenhagen and Oxford (Table 1).

As described earlier, either of the complementary DNA strands can act as templates for PCR, as any single miscoding lesion event can produce two observable phenotypes post-PCR. It has been argued that since the chemical events required to generate direct G → A and T → C transitions are biochemically unlikely, any G → A and T → C damage that is observed on a particular DNA strand must have originated on the complementary strand as C → U and A → H events, respectively (GILBERT *et al.* 2003b). Others have questioned this

TABLE 2
Primers and PCR conditions

Species/gene ^a	Primer name ^b	Primer sequence (5'–3') ^c	Annealing temperature/ cycles ^d	Source ^e
Woolly rhino/ 12S rDNA	21pheF 684-12Sr	AAAGCAAGGCATTGAAAATGCCTAGATGA GGCGGTATATAGGCTGAATT	55°/×40	TOUGARD <i>et al.</i> (2001)
Woolly rhino/ 12S rDNA	650-12sF 1085valR	CCGATAAACCCCGATAAACC TGAAATCTCCTGGGTGTAAGC	56°/×40	This study
Woolly rhino/ Cytochrome <i>b</i>	14228glu-2F 14596cytB	ACCAATGACATGAAAAATCATCGTT TTTCAGGTTTCTAGGAAGGTGT	52°/×40	This study
Woolly rhino/ Cytochrome <i>b</i>	14279cytB 15107cytB	ATGACTAACATCCGCAAATCCC GGGATGGATCGTAGGATTGCGTA	66°/×40	This study
Woolly rhino/ Cytochrome <i>b</i>	14912cytB 15446thrR	CCAACATAGACAAAATCCC CCTTTTCTGGTTTACAAGACC	52°/×40	This study
Woolly rhino/ Real time PCR 1	WR_QPCR_415F WR_QPCR_484R	ACGTCCTACCATGAGGCCAA GGGATAGCTGAGAGAAGGTTTGTG	55°/×40	This study
Woolly rhino/ Real time PCR 2	WR_QPCR_411F WR_QPCR_476R	GGCTACGTCCTACCATGAGGC TGAGAGAAGGTTTGTGATGACTGTG	55°/×40	This study
Woolly rhino/Numt-1	66WR-PseudoF 405WR-PseudoR	GTAAGCATATGGTAAGCAC GCTACACTTTGGTTTATCCAACCTCC	55°/×50	This study
Pig / Mt. control region	L15387 H648n	CTCCGCCATCAGCACCCAAAAG GCTYATATGCATGGGGACT	56°/×40	LARSON <i>et al.</i> (2005)
Pig / CD45	PigEx9f PigEx9r	GAGAAATACATGGATATCCCTG CTGGAGGTGTCTCTAAGAGG	56°/×40	This study
Lion / ATP8 and num.	ATP8_1F ATP8_3R	GCCACAGTTAGATACATC GAGGTGAATAGATTTTCGTTTC	56°/×40	I. BARNES, personal communication
Moa / Mt. control region	185fm-CR 294rm-CR	GTACATTCCCTGCATTGGCTC GCGAGATTGACAGTACG	55–60°/×40	BUNCE <i>et al.</i> (2003)
Moa / Mt. control region	262fm-CR 441rm-CR	GCGAAGACTGACTAGAAGC CGCATACCGGGTCTGTTTATGC	55–60°/×40	BUNCE <i>et al.</i> (2003)
Moa / KW1 gene (sex linked)	Kw1-185f Kw1-260r	GGCYRYTGCCCTCAGAAATTACAG ATGCTATACTGCTTTAACAGA	52–55°/×40–50	BUNCE <i>et al.</i> (2003)
Moa / ADH gene	Adh-230f Adh-290r	GAGGAATTAGCTYRTTAGCTGTG GGTTAACACTTTGCCAGTGG	52–55°/×40–50	BUNCE <i>et al.</i> (2003)

^a Specimen name and sequence region amplified.

^b Primer name.

^c Primer sequence.

^d Annealing temperature/ no. of PCR cycles.

^e Primer source.

hypothesis due to the limited knowledge of DNA damage in fossil remains (PÄÄBO *et al.* 2004). We have therefore decided to follow the approach by HANSEN *et al.* (2001) and HOFREITER *et al.* (2001a) and not distinguish between the different phenotypes of TS1 (A → G/T → C) and TS2 (C → T/G → A).

Total sequence heterogeneity (TSH) was calculated as the probability of observing transitions in a single position following the formula $TSH = 1/n$, where 1 is the total number of observed substitutions and n is the total number of nucleotides examined (after GILBERT *et al.* 2003a; Table 3; Figure 1). Two sequences showing PCR artifacts in the form of “jumping PCR” are marked in the clone data sets (supplemental material S4–S40 at <http://www.genetics.org/supplemental/>) as CHI for chimeric. These sequences were not included in the analysis.

Type 1 and type 2 transitions were calculated in a similar fashion, resulting in three values, p_{TSH} , p_{TS1} , and p_{TS2} , for each of the $2 \times 23 = 46$ clone data sets. Transitions were scaled by multiplying with the AT:GC ratio of the specific gene region to compensate for nucleotide composition bias. Subsequently, p_i , $i = TSH, TS1, \text{ and } TS2$, were transformed using $r_i =$

$-\log(1 - p_i)$, where $r_i = a_i T$, T is the age of the specimen, and a_i is the average damage rate per unit time. The r_i , $i = TSH, TS1, \text{ and } TS2$, are referred to as the TSH, TS1, and TS2 rates, respectively.

Pearson's correlation coefficient ρ was calculated between (i) TSH and TS1 rates; (ii) TSH and TS2 rates; (iii) TS1 and TS2 rates; (iv) age of specimens and TSH rate; (v) age of specimens and TS1 rate; (vi) age of specimens and TS2 rate; (vii) age and the ratio of TS2 rate to that of TS1 (TS2/TS1; excluding cases where $r_{TS1} = 0$); and (viii) TSH rate and TS2/TS1 (excluding cases where $r_{TS1} = 0$). The correlation analysis was performed for all 46 clone sets and separately for the 23 nuclear clones and the 23 mitochondrial clones.

A t -test was performed to investigate whether TSH rates in nuDNA clones were significantly different from those in mtDNA clones. Data were further divided into three groups according to the environment of preservation: (i) permafrost specimens ($n = 11$); (ii) cave and dry swamp specimens ($n = 7$), which were originally excavated, although some had subsequently been stored in museums for years; and (iii) museum

TABLE 3
Total sequence heterogeneity

Substitutions ^a	A → G/T → C	A → T/T → A	A → C/T → G	C → T/G → A	C → G/G → C	C → A/G → T	Total
No. of substitutions in mtDNA	125.00	33.00	21.00	294.73	20.05	31.58	525.36
%	23.79	6.28	4.00	56.10	3.82	6.01	100
No. of substitutions in nuDNA	42.00	9.00	3.00	218.10	0.00	6.35	278.44
%	15.08	3.23	1.08	78.33	0.00	2.28	100

^aThe sum of the different substitutions for all 23 mitochondria and nuclear data sets. Substitutions originating from a C or a G have been corrected for the AT:GC ratio for each gene region.

specimens ($n = 5$), which had always been stored in museums. Using *t*-tests it was investigated whether TSH rates, TSH rates divided by age, and the TS2/TS1 rates ratio differ among the three groups. Finally, it was investigated (using a chi-square test) whether the observed patterns of base transitions in mtDNA and nuDNA sequences were similarly distributed. All tests were performed at the 1% significance level.

RESULTS AND DISCUSSION

In this study nuclear and mitochondrial DNA were PCR amplified, cloned, and sequenced from a variety of faunal remains and combined with previously published clone data to compare the frequencies and types of miscoding lesion damage. As miscoding lesions are not the only factor that might influence intracolon heterogeneity, it is important to take into account other sources, such as innate DNA polymerase misincorporation errors, natural sequence heterogeneity, and variation in the number of starting template molecules for PCR (HANSEN *et al.* 2001). We believe these factors are unlikely to have influenced the data in a significant fashion for the following reasons:

- i. The majority of the clone sequences analyzed were generated using the proofreading DNA polymerase enzyme Platinum Taq High Fidelity, an enzyme with an innate error rate on good quality DNA of 2.0×10^{-6} – 6.5×10^{-7} /nucleotide/cycle (FLAMAN *et al.* 1994; ANDRÉ *et al.* 1997). Although some have speculated that aDNA extracts might increase the misincorporation rates of some polymerases (*e.g.*, PUSCH and BACHMANN 2004), our enzyme has been shown to retain its low misincorporation on DNA amplified from aDNA extracts (GILBERT *et al.* 2003b). Therefore, regular sequence errors are likely to account for only minor amounts of the calculated sequence heterogeneity, although we cannot completely exclude them from the data sets (*e.g.*, errors generated during bacterial colony growth or sequencing).
- ii. It is possible that the number of starting template molecules in the PCR reactions might influence the observed sequence heterogeneity. In the most extreme case, a PCR that starts off a single template

molecule will not demonstrate any variation and hence appear damage free. However, for the sequence data generated in this study we are confident that the PCR reactions started from considerably more than a single template molecule, since dilutions up to 1/50 of the extracts prior to amplification still yielded amplification products for both the mtDNA and the nuDNA markers, the clones showed variation, and the PCR products quantified showed starting template numbers in the 10^2 – 10^4 range (supplemental material at <http://www.genetics.org/supplemental/>).

- iii. It seems unlikely that single-substitution heteroplasmy and recombination in the mtDNA data sets influence the observed heterogeneity due to both their reported rarity in coding regions of mammalian mtDNA and their apparent absence in complete mtDNA studies (including D-loop sequences) of pigs, moa, cats, mammoths, and rhinoceroses (GHIVIZZANI *et al.* 1993; LOPEZ *et al.* 1996; XU and ARNASON 1997; URSING and ARNASON 1998; LIN *et al.* 1999; COOPER *et al.* 2001; J. KRAUSE, personal communication).

However, we cannot exclude the possibility that adverse amplifications of nuclear alleles, pseudogenes, and other gene duplicates might contribute to the observed variation in the nuclear data sets (for a discussion see below and GREENWOOD *et al.* 1999, 2001). Therefore, with regard to the validity of the data, we believe that the observed heterogeneity in the mtDNA clones can be explained predominantly by damage, while the variation observed in the nuDNA data sets may have arisen from a combination of miscoding lesions and other factors. Despite this, only a minority of the specimens (8 of 23) exhibited higher TSH in the nuDNA sequences than in the mtDNA sequences ($P = 17\%$, Figure 1). However, no significant differences in TSH levels between nuDNA and mtDNA sequences ($P = 17\%$) were observed, suggesting that nuDNA miscoding lesion damage is less than or equal to that of mtDNA, despite a lower number of cellular copies. Intriguingly, nuDNA appears to be more limited by amplification length than mtDNA (BUNCE *et al.* 2003; POINAR *et al.* 2003). This may represent a simple case of template quantity, whereby

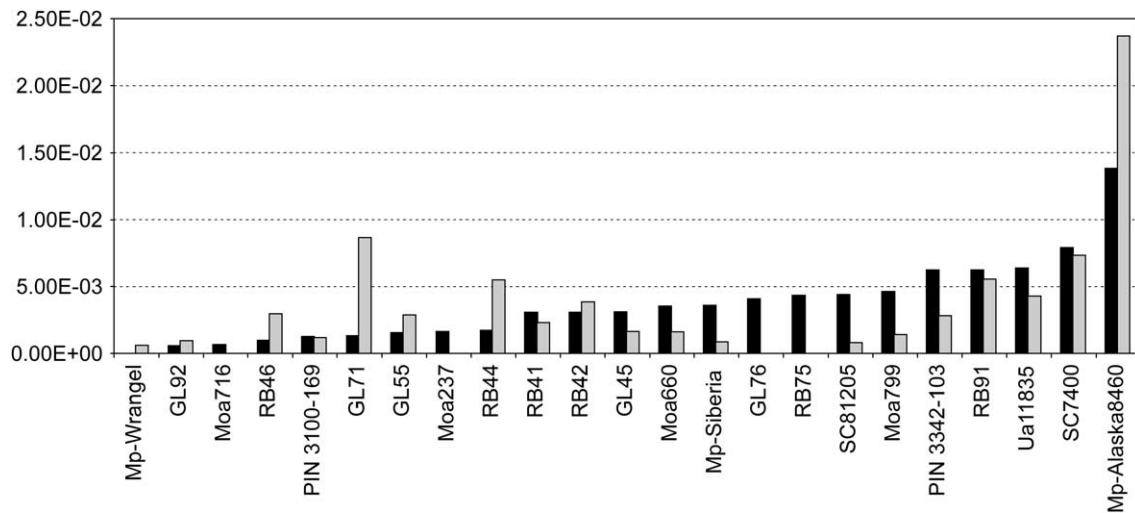


FIGURE 1.—Total sequence heterogeneity observed in the mtDNA and nuDNA clone sequences from the 23 specimens (see Table 1). Solid bars, mtDNA total sequence heterogeneity. Shaded bars, nuDNA total sequence heterogeneity.

the abundance of mtDNA makes it more likely that long undamaged molecules exist. Alternatively, the nucleosome core (146 bp of nuDNA wrapped around a histone octamer) may represent a key “preservation unit” whereby strand breaks may be common in the linker regions between adjacent cores. Finally, the proximity of chromosomal proteins (*e.g.*, histones) to the DNA increases the chance of forming protein–DNA crosslinks, structures that could easily act as polymerase blocks during amplification.

A significant difference was observed in the distributions of miscoding lesion “types” in the mtDNA and nuDNA sequences ($P < 0.1\%$; Table 3). Overall the frequency of type 1 transitions ($A \rightarrow G/T \rightarrow C$) is lower in the nuDNA (15%) than in the mtDNA (24%) and the frequency of type 2 transitions ($C \rightarrow T/G \rightarrow A$) is higher (78% *vs.* 56%). This implies that different types of damage could occur at different rates in the mitochondria and the nucleus. The fact that mtDNA is not complexed with histone proteins could make it more susceptible to different types of oxidative and/or hydrolytic damage. However, at this stage we cannot rule out that the observed differences are due to other factors such as natural variation in the nuDNA.

Although a number of aDNA studies attribute the presence of type 1 transitions to postmortem damage (*e.g.*, HANSEN *et al.* 2001; GILBERT *et al.* 2003b), other studies (*e.g.*, HOFREITER *et al.* 2001a; PÄÄBO *et al.* 2004) argue that their existence is an artifact of regular DNA polymerase errors. The positive correlations observed in this study between TSH and the number of type 1 ($\rho = 0.48$; Figure 2A) and type 2 transitions ($\rho = 0.66$; Figure 2B) and between TS1 and TS2 rates ($\rho = 0.45$; Figure 2C) make it difficult to explain the type 1 transitions solely on the basis of DNA polymerase errors. Furthermore, we find no obvious correlation between the rate of type 1 transitions and the type of DNA polymerase en-

zyme used (supplemental material at <http://www.genetics.org/supplemental/>). However, we do observe some discrepancy in the levels of type 1 relative to type 2 transitions compared to previous observations (GILBERT *et al.* 2003b). In this study, we observe a total of 167 and 512 ($\sim 1:3$) type 1 and type 2 events (Table 3), respectively (counts are adjusted for base composition), which is lower than the 177 and 366 ($\sim 1:2$) type 1 and type 2 events observed by GILBERT *et al.* (2003b). Additionally, only 7 of the 46 data sets investigated (both mtDNA and nuDNA) show more type 1 than type 2 transitions (supplemental material at <http://www.genetics.org/supplemental/>) compared to 26 of 65 human mtDNA data sets studied by GILBERT *et al.* (2003b). However, our observed type 1: type 2 ratio is higher than that reported by HOFREITER *et al.* (2001a). When distinguishing between consistent and singleton substitutions, HOFREITER *et al.* (2001a) report consistent changes to be only type 2 events, and the ratio for the remaining singletons to be 44 type 1 to 282 type 2 ($\sim 1:6$) (M. HOFREITER, unpublished data).

The correlation analyses show a clear overall bias toward type 2 transitions (in both nuDNA and mtDNA templates) with increasing levels of total sequence heterogeneity ($\rho = 0.42$; $P = 0.7\%$; Figure 2D). This pattern has previously been reported for human D-loop sequences (GILBERT *et al.* 2003b), although it has been argued that in this case the pattern could be partially or completely explained by contamination, which is especially problematic in studies of human aDNA (PÄÄBO *et al.* 2004). Our results therefore confirm the general presence of a type 2 damage bias in mtDNA and demonstrate that this observation also holds true for nuDNA. As such, the results corroborate the previously noted observation (GILBERT *et al.* 2003b) that, under equal environmental conditions, type 2 transitions occur at a faster rate than type 1 transitions. This in turn indicates that

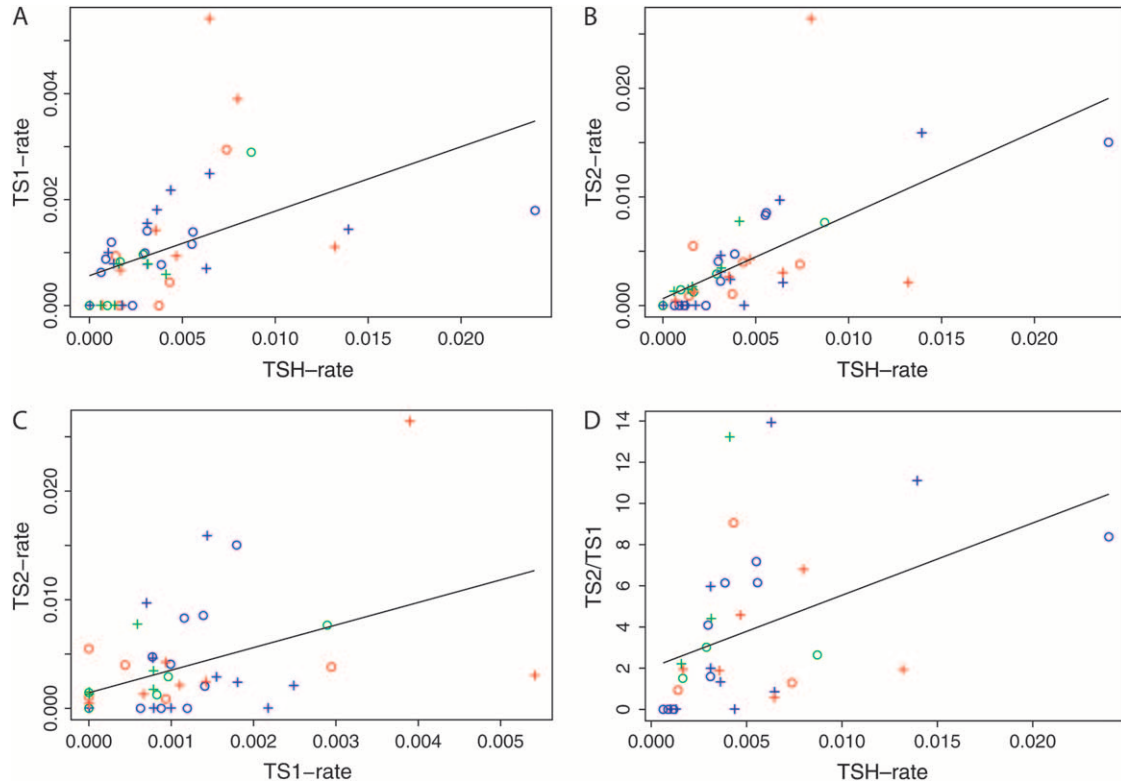


FIGURE 2.—Correlation analyses. Colors indicate the preservation environment: blue, permafrost; red, cave and swamp; and green, museum samples. + denotes mtDNA sequences and O denotes nuDNA sequences. (A) Type 1 transitions (TS1) as a function of TSH. (B) Type 2 transitions (TS2) as a function of TSH. (C) TS1 as a function of TS2. (D) TS2/TS1 as a function of TSH.

deamination of cytosine and its homolog 5-methyl cytosine to uracil and adenine is the dominant type of miscoding lesion in both mtDNA and nuDNA sequences from fossil remains.

The observed lack of correlation between DNA damage and age of specimens was not unexpected (TSH: $\rho = 0.29$, $P = 2.3\%$; TS1: $\rho = 0.15$, $P = 16\%$; TS2: $\rho = 0.14$, $P = 17\%$; TS2/TS1: $\rho = -0.15$, $P = 2.1\%$; supplemental material at <http://www.genetics.org/supplemental/>) as numerous studies have demonstrated that preservation conditions rather than age determine rates of DNA degradation (PÄÄBO 1989; LINDAHL 1993; HÖSS *et al.* 1996; POINAR *et al.* 1996; KUMAR *et al.* 2000; HOFREITER *et al.* 2001a; SMITH *et al.* 2001; GILBERT *et al.* 2003b; PÄÄBO *et al.* 2004; WILLERSLEV *et al.* 2004a; WILLERSLEV and COOPER 2005). More surprising is the apparent lack of significant differences among the three groups (permafrost, cave, and museum) for TSH rate and the ratio of TS2/TS1 (P -values $>20\%$; Table 4), because these environments represent different temperature regimes of storage that are known to influence damage rates (Höss *et al.* 1996; SMITH *et al.* 2001; WILLERSLEV *et al.* 2004a). However, the museum group differed from the other two groups when comparing TSH rate/age (permafrost, $P < 1\%$; cave, $P < 1\%$), while there was no difference between the permafrost and the cave groups ($P = 12\%$). This is likely due to long-term storage at room temperature after excavation of most of the mam-

moth specimens (GREENWOOD *et al.* 1999, 2001). Importantly, the museum samples (all museum samples are from pigs <130 years of age) show significantly higher overall sequence heterogeneity than the environmental samples, suggesting that the given museum storage conditions are not optimal for DNA preservation. Given that museum/herbarium material is an important source of aDNA, it is concerning that little research has been conducted to investigate how to maximize biomolecular preservation. If indeed DNA damage and/or degradation accumulates as the result of suboptimal storage conditions in museums (as has been observed empirically by many researchers), then alterations to current sample storage practices should be investigated.

In summary, this study demonstrates that there is no significant evidence for nuDNA sequences being more

TABLE 4
Results of *t*-test

Test ^a	<i>t</i> -value	<i>P</i> (%)
Permafrost <i>vs.</i> caves	0.18	85
Permafrost <i>vs.</i> museum	1.31	20
Caves <i>vs.</i> museum	1.22	24

^a The total sequence heterogeneity for specimens preserved under different conditions (permafrost, caves, or museum storage) is compared.

prone to miscoding lesions than mtDNA sequences despite the large discrepancy in cellular copy numbers. The data also suggest that deamination of cytosine (and 5-methyl cytosine) is the most frequent type of miscoding lesion in mtDNA and nuDNA sequences from fossil remains, although the rate at which they are affected may differ. Additionally, a bias toward deamination of cytosine relative to type 1 transitions with an overall increase in damage appears to apply to both ancient mtDNA and nuDNA sequences. Finally, we note that the findings reported in this study are limited by the sample size, and as more relevant data accumulate, future studies might be able to focus on other important questions, such as whether correlations exist between specimen type and state of DNA damage.

In conclusion, in the decade ahead, the field of aDNA will increasingly focus on nuclear genes from fossil and archival material. However, as the aDNA community embarks in this new direction, considerable care needs to be exercised to ensure that authentic sequences are generated. In the past two decades, many aDNA articles have been published in which the mtDNA data have turned out to be contaminated, pseudogenes, and/or modified by damage (for recent reviews see PÄÄBO *et al.* 2004; WILLERSLEV and COOPER 2005). Therefore, we find it important to highlight briefly the variety of factors that need to be considered when amplifying and analyzing ancient nuDNA.

To gain a complete, unbiased appraisal of the damage spectrum in both ancient nuclear and mitochondrial DNA, further studies should attempt to generate PCR products of the same length with an equal number of starting template molecules. Additionally, when designing PCR assays for ancient nuclear loci, restricting amplicon size will both increase the chance of successful amplification and maximize the number of starting template molecules. Importantly, sequence heterogeneity in the clone data might in fact represent real allelic variation. In this study we cannot rule out that some sites interpreted as damaged might indeed represent real allelic differences (*e.g.*, the fast-evolving CD45 gene seen in the pig data). For the moa data, however, one of the nuclear genes (*kw1*) is sex linked and therefore will have no allelic variation. Allelic variation in combination with low starting template numbers can also lead to cases of "allelic dropout" whereby one allelic form is amplified preferentially over another, which calls for reproducibility of results (TABERLET *et al.* 1996; MORIN *et al.* 2001).

In the same way that nuclear mitochondrial insertions (numts) have caused the misinterpretation of mitochondrial phylogenies (see WILLERSLEV and COOPER 2005), nuclear pseudogenes and gene duplications can cause problems in the interpretation of nuDNA sequences. Complete genomes are now available for a variety of organisms, making it possible to screen for the presence of duplicated genes. However, in cases where a genetic background is not well characterized, there is a consid-

erable risk of coamplifying a functional duplicated gene or a nonfunctional pseudogene.

Finally, contamination has been a major problem in the field of aDNA (PÄÄBO *et al.* 2004; WILLERSLEV and COOPER 2005). When amplifying mitochondrial templates, a contaminating sequence can often be identified; for example, a D-loop sequence from contemporary humans is readily distinguishable from that of Neanderthals (*e.g.*, SERRE *et al.* 2004b). When amplifying highly conserved nuclear targets, contamination will not be so easy to spot and can easily be mistaken for allelic variation, and consequently it might be difficult to distinguish between exogenous and endogenous DNA sequences.

We advocate that as the field of aDNA moves into amplifying nuclear targets, factors such as damage, amplicon size, allelic variation, low template copy numbers, and contamination need to be taken into account.

We are grateful to Hendrik Poinar, Johannes Krause, Tina Brand, and Martin B. Hebsgaard for help and discussion; Andrei V. Sher and Ross Macphee for providing the woolly rhinoceros and mammoth samples; and Michael Hofreiter for providing unpublished sequence data. J.B. and E.W. were supported by the Carlsberg Foundation of Denmark, the National Science Foundation of Denmark, and the Wellcome Trust. C.W. was supported by the Danish Cancer Society and the Carlsberg Foundation. R.B. was supported by the Biotechnology and Biological Sciences Research Council and Natural Environment Research Council.

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