More on Contamination: The Use of Asymmetric Molecular Behavior to Identify Authentic Ancient Human DNA

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Authentication of ancient human DNA results is an exceedingly difficult challenge due to the presence of modern contaminant DNA sequences. Nevertheless, the field of ancient human genetics generates huge scientific and public interest, and thus researchers are rarely discouraged by problems concerning the authenticity of such data. Although several methods have been developed to the purpose of authenticating ancient DNA (aDNA) results, while they are useful in faunal research, most of the methods have proven complicated to apply to ancient human DNA. Here, we investigate in detail the reliability of one of the proposed criteria, that of appropriate molecular behavior. Using real-time polymerase chain reaction (PCR) and pyrosequencing, we have quantified the relative levels of authentic aDNA and contaminant human DNA sequences recovered from archaeological dog and cattle remains. In doing so, we also produce data that describes the efficiency of bleach incubation of bone powder and its relative detrimental effects on contaminant and authentic ancient DNA. We note that bleach treatment is significantly more detrimental to contaminant than to authentic aDNA in the bleached bone powder. Furthermore, we find that there is a substantial increase in the relative proportions of authentic DNA to contaminant DNA as the PCR target fragment size is decreased. We therefore conclude that the degradation pattern in aDNA provides a quantifiable difference between authentic aDNA and modern contamination. This asymmetrical behavior of authentic and contaminant DNA can be used to identify authentic haplotypes in human aDNA studies.

Introduction

Contaminating modern human DNA hampers studies on ancient human DNA. Ancient and modern haplotypes and alleles are often identical and there is no conclusive way of identifying contaminating modern DNA. As the contamination mainly derives from exogenous DNA in the material (Richards et al. 1995; Hofreiter et al. 2001; Malmström et al. 2005), replication in an independent laboratory will not eliminate the problem. Contaminant DNA largely behaves like ancient DNA (aDNA) in amplicon cloning and often yields a singular sequence or sequence variation that is the result of degradation of one original contaminant sequence (Malmström et al. 2005; Sampietro et al. 2006). Thus, authentication procedures designed to avoid and detect contaminating DNA in ancient tissue (Cooper and Poinar 2000) are of little help in studies on ancient human remains. The problem was acknowledged as early as a decade ago (Handt et al. 1994; Richards et al. 1995; Handt et al. 1996), and more recently there has been several reports on different aspects of contamination in ancient human DNA studies (Bandelt 2005; Gilbert et al. 2005; Kemp and Smith 2005; Malmström et al. 2005; Salamon et al. 2005; Bouwman et al. 2006; Sampietro et al. 2006). Although the problem is widely recognized, there remains a strong desire to work with genetics in ancient humans nevertheless (Dalton 2005; Haak et al. 2005; Sampietro et al. 2005).

It has been previously suggested that a comparison of the degree of degradation between different sources of DNA in an ancient sample (i.e., between the authentic source DNA and the contaminant sources of DNA) might be used as a tool to authenticate ancient DNA. The argument has been referred to as "appropriate molecular behavior" (Cooper and Poinar 2000). The logic behind this argument is as follows. Postmortem, DNA molecules degrade as a loose function of temperature and time (cf. Smith et al. 2001). Thus for any given source of DNA, over time there will be the generation of an increased number of short fragments and a decrease in the number of longer fragments. As contaminant sources of DNA are younger in age than the true endogenous DNA sequences, it is to be expected that the relative levels of short to long DNA fragments derived from the contaminant should be lower than for the endogenous DNA. Furthermore, the average fragment size of modern contaminant DNA should be higher in comparison to ancient degraded DNA (Noonan et al. 2005).

In this paper, we quantify DNA fragments of different sizes derived from contaminant human and authentic ancient dog and cow DNA. Prior to DNA extraction, the powdered samples were pretreated with bleach, a method which has been proposed as an effective means to minimize the carryover of contaminant DNA sequences from the bone in to the final DNA extract (Kemp and Smith 2005; Salamon et al. 2005). As part of this study, we investigate the efficiency of the decontamination method, through comparison of the data generated here with data previously generated from extractions on the same specimens, performed in the absence of the bleach pretreatment. We use the extracted DNA to measure the quantitative relation between long and short contaminant fragments and authentic DNA. We predict that a decrease in targeted fragment length will result in a significantly higher proportion of authentic DNA.

Material and Methods

We extracted, amplified, and sequenced DNA from 23 prehistoric dog bones and teeth that had yielded DNA in a previous study (Malmström et al. 2005) and a bone and a tooth from 2 additional dogs (table 1) together with 9 extraction controls amplified in duplicates for all fragments

Key words: contamination, ancient DNA, authentication.

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Table 1
Number of Human and Dog mtDNA Templates in Each Dog Specimen

				Human DNA[a]				Dog DNA[a]		
Sample	Locality	Element	Age	H148	Replication H148[b]	H112	D152	Replication D152	D111	D93
1	Korsnäs	Bone	Neolithic	13 ± 19	NA	20 ± 1	0 ± 0	NA	0 ± 0	0 ± 0
2	Korsnäs	Bone	Neolithic	9 ± 13	105 ± 100	8 ± 12	0 ± 0	1680	13 ± 18	27 ± 2
3	Korsnäs	Bone	Neolithic	7 ± 9	33 ± 35	68 ± 8	0 ± 0	1252 ± 1263	0 ± 0	0 ± 0
4	Korsnäs	Bone	Neolithic	10 ± 15	121	29 ± 41	188 ± 13	839	1437 ± 114	2073 ± 113
5	Korsnäs	Bone	Neolithic	8 ± 1	NA	103 ± 57	0 ± 0	NA	0 ± 0	0 ± 0
6	Jettböle	Bone	Neolithic	19 ± 13	NA	23 ± 12	0 ± 0	NA	24 ± 34	39 ± 9
7	Jettböle	Bone	Neolithic	0 ± 0	NA	99 ± 39	0 ± 0	NA	0 ± 0	0 ± 0
9	Jettböle	Bone	Neolithic	94 ± 65	NA	65 ± 6	0 ± 0	NA	0 ± 0	0 ± 0
10	Källsveden	Bone	Neolithic	20 ± 2	NA	9 ± 12	0 ± 0	NA	0 ± 0	0 ± 0
13	Ajvide	Bone	Neolithic	20 ± 11	NA	33 ± 0	49 ± 11	NA	471 ± 276	920 ± 234
14	Ajvide	Bone	Neolithic	31 ± 2	NA	26 ± 15	0 ± 0	NA	0 ± 0	0 ± 0
15	Ajvide	Bone	Neolithic	55 ± 20	56	35 ± 30	117 ± 87	386	1344 ± 319	2222 ± 458
16	Ajvide	Bone	Neolithic	40 ± 26	129	51 ± 22	129 ± 39	744	1117 ± 99	1852 ± 27
17	Ajvide	Bone	Neolithic	10 ± 14	28	79 ± 10	140 ± 9	NA	430 ± 8	54 ± 25
18	Ajvide	Bone	Neolithic	59 ± 23	28	24 ± 8	15 ± 9	NA	155 ± 40	255 ± 95
19	Ajvide	Bone	Neolithic	30 ± 10	1268	66 ± 22	160 ± 46	715	565 ± 71	781 ± 239
20	Ajvide	Teeth	Neolithic	205 ± 62	NA	241 ± 29	532 ± 111	NA	1928 ± 324	2598 ± 269
21	Ajvide	Teeth	Neolithic	30 ± 43	22	123 ± 49	1681 ± 315	NA	7664 ± 216	14718 ± 145
22	Ajvide	Teeth	Neolithic	4 ± 6	21	74 ± 50	504 ± 108	2034	2212 ± 310	3906 ± 309
23	Ajvide	Teeth	Neolithic	0 ± 0	NA	52 ± 10	852 ± 126	NA	3163 ± 414	5415 ± 614
24	Ajvide	Teeth	Neolithic	9 ± 13	NA	15 ± 1	0 ± 0	NA	156 ± 1	336 ± 55
27	Skara B	Bone	Medieval	18 ± 26	131	18 ± 8	168 ± 5	NA	651 ± 148	961 ± 62
28	Stockholm	Bone	Medieval	21 ± 15	NA	59 ± 21	93 ± 0	NA	291 ± 104	499 ± 52
30	Visby	Teeth	Neolithic	9 ± 13	NA	15 ± 21	58 ± 1	NA	348 ± 116	599 ± 68
31	Ire	Bone	Neolithic	14 ± 3	NA	30 ± 10	0 ± 0	NA	0 ± 0	25 ± 35

[a] The average number (±SD) of mtDNA templates from duplicate amplification products quantified with real-time PCR.
[b] SD is given for the independent replications that were done more than once.

and 4 polymerase chain reaction (PCR) controls, also for all fragments according to previously published protocols (Yang et al. 1998; Malmström et al. 2005). The majority of this specific material was excavated more than 10 years ago, and the previous study showed that the DNA was of good quality (Malmström et al. 2005). However, as the material was believed to be severely contaminated with modern human DNA, 2 modifications were added to the extraction protocols. After thorough sandpaper polishing, bones and teeth were incubated in 0.1 M HCl for 5 min, washed 3 times in ddH_2O (DNA free ELGA grade) and once in 95% EtOH. Following powdering, the samples were soaked in 0.5% bleach for 15 min and washed 3 times in LiChrosolv water (Merck, Darmstadt, Germany) prior to DNA extraction. The HCl and bleach treatment had not been used in the previous study. Two fragment sizes (148 and 112 bp, denoted H148 and H112, respectively) were targeted for amplification with primers (table 2) specific for the human mitochondrial D-loop, and similar respectively) were targeted with primers (table 2) specific

Table 2
Primers and Probes for Human and Dog mtDNA and for Cattle/Human 16S

	Primer/Probe Name	Primer/Probe Sequence 5′–3′	Annealing Temperature
1	Human F	CTGCCAGCCACCATGAATATT	
2	Human MGB	TACCATAAATACTTGACCACCTG	
3	Human R 112 bp	TGCTGTACTTGCTTGTAAGCATGG	59
4	Human R 148 bp	GGAGTTGCAGTTGATGTGTGATAGT	59
5	Dog F	CCATCAGCACCCAAAGCTG	
6	Dog MGB	TTCTTCTTAAACTATTCCCTGACAC	
7	Dog R 93 bp	ATACTGACATAGCACAGTAGGGGTGAT	59
8	Dog R 111 bp	AGAAGGGTTTACCTGGAGATACTGACA	59
9	Dog R 152 bp	ATGGGGCAAACCATTAATGC	59
10	Cow/human F bio[a]	AGGGATAACAGCGCAATCCTATTC	
11	Cow/human Pyro A/T	CCCTATTGTRGATATGGACT	
12	Cow/human R 70 bp	TGATCCAACATCGAGGTCGTAAAC	47
13	Cow/human R 124 bp	CTTTAATCGTTGAACAAACGAACC	52
14	Cow/human R 178 bp	WARTAGATAGAAACCGACCTGG	52
15	Cow/human R 180 bp	YGWARTAGATAGAAACCGACCTGG	52

[a] Primer number 11 was used was used as internal pyrosequencing primer with the following dispensation order: GCTACGATA.

Table 3
Proportion of Cattle Fragments (A status) in Each Cattle Sample without Bleach Treatment and with Treatment of different Bleach Concentrations

Sample	Locality	Century	%70 bp (A)	%124 bp (A)	%178 bp (A)	%180 bp (A)
Without Bleach Treatment						
MS2 e1	Marstrand	18th	98	100	97	97
MS2 e2	Marstrand	18th	97	100	96	93
MS3 e1	Marstrand	18th	98	100	92	96
MS3 e2	Marstrand	18th	100	100	96	98
MS4 e1	Marstrand	18th	97	97	90	97
MS4 e2	Marstrand	18th	97	97	93	NA
MS6 e1	Marstrand	18th	98	97	96	97
MS6 e2	Marstrand	18th	76	97	94	97
MS7 e1	Marstrand	18th	97	100	96	96
MS7 e2	Marstrand	18th	96	94	90	90
MS8 e1	Marstrand	18th	98	98	96	97
MS8 e2	Marstrand	18th	97	100	97	97
MS9 e1	Marstrand	18th	96	94	78	93
MS9 e2	Marstrand	18th	95	91	NA	90
MS10 e1	Marstrand	18th	100	97	97	97
MS10 e2	Marstrand	18th	100	97	96	96
S1 e1	Skara	13th	86	76	NA	62
S1 e2	Skara	13th	95	83	NA	85
S2 e1	Skara	13th	27	17	13	11
S2 e2	Skara	13th	27	17	0	22
S3 e1	Skara	13th	91	90	76	81
S3 e2	Skara	13th	87	92	NA	100
S4 e1	Skara	13th	44	NA	19	27
S4 e2	Skara	13th	67	73	16	46
S5e1	Skara	13th	70	55	62	67
S5 e2	Skara	13th	83	83	78	84
S6 e1	Skara	13th	82	83	76	75
S6 e2	Skara	13th	93	95	87	91
S7 e1	Skara	13th	64	NA	NA	39
S7 e2	Skara	13th	72	69	57	66
S8 e2	Skara	13th	59	NA	NA	31
S9e1	Skara	13th	52	34	NA	32
S9e2	Skara	13th	50	NA	NA	28
S10e1	Skara	13th	79	NA	NA	61
S10e2	Skara	13th	20	NA	NA	NA
S11e1	Skara	13th	94	95	92	91
S11e2	Skara	13th	94	94	92	95
S12e1	Skara	13th	NA	NA	88	91
S12e2	Skara	13th	96	97	94	97
S13e1	Skara	13th	65	63	37	69
S13e2	Skara	13th	39	37	19	32
S14e1	Skara	13th	42	34	NA	28
S14e2	Skara	13th	58	46	38	34
S15e1	Skara	13th	35	33	43	14
S15e2	Skara	13th	48	55	57	50
S16e1	Skara	13th	93	94	89	81
S16e2	Skara	13th	NA	NA	39	42
With Treatment of different Bleach Concentrations						
No Bleach						
ML4 e1	Marstrand	18th	95	97	98	97
ML4 e2	Marstrand	18th	93	96	97	96
ML9 e1	Marstrand	18th	96	97	99	97
ML9e2	Marstrand	18th	96	96	97	93
S19 e1	Skara	13th	NA	82	NA	93
S19 e2	Skara	13th	78	95	94	95
S20 e1	Skara	13th	NA	NA	NA	NA
S20 e2	Skara	13th	80	85	84	76
S21 e1	Skara	13th	90	93	88	90
S21 e2	Skara	13th	93	96	96	94
S22 e1	Skara	13th	95	94	NA	95
S22 e2	Skara	13th	93	94	95	93
S23 e1	Skara	13th	89	82	76	78
S23 e2	Skara	13th	90	2	87	82
S24 e1	Skara	13th	NA	NA	NA	NA
S24 e2	Skara	13th	NA	NA	NA	NA

Table 3
Continued

Sample	Locality	Century	%70 bp (A)	%124 bp (A)	%178 bp (A)	%180 bp (A)
S25 e1	Skara	13th	87	89	80	78
S25 e2	Skara	13th	85	86	87	79
S26 e1	Skara	13th	93	94	94	94
S26 e2	Skara	13th	94	94	93	93
0.5% Bleach						
ML4 e1	Marstrand	18th	88	97	97	97
ML4 e2	Marstrand	18th	84	93	92	NA
ML9 e1	Marstrand	18th	96	97	97	97
ML9e2	Marstrand	18th	96	96	97	97
S19 e1	Skara	13th	87	95	78	88
S19 e2	Skara	13th	85	97	95	95
S20 e1	Skara	13th	NA	93	70	81
S20 e2	Skara	13th	83	95	94	97
S21 e1	Skara	13th	89	95	93	88
S21 e2	Skara	13th	83	94	82	85
S22 e1	Skara	13th	80	96	93	95
S22 e2	Skara	13th	95	97	97	98
S23 e1	Skara	13th	92	94	93	94
S23 e2	Skara	13th	91	96	93	97
S24 e1	Skara	13th	NA	92	87	90
S24 e2	Skara	13th	NA	88	64	NA
S25 e1	Skara	13th	86	93	90	94
S25 e2	Skara	13th	87	96	100	98
S26 e1	Skara	13th	95	94	96	94
S26 e2	Skara	13th	92	96	94	95
3% Bleach						
ML4 e1	Marstrand	18th	55	95	96	93
ML4 e2	Marstrand	18th	100	82	NA	NA
ML9 e1	Marstrand	18th	96	97	97	96
ML9e2	Marstrand	18th	94	100	97	97
S19 e1	Skara	13th	94	95	98	97
S19 e2	Skara	13th	92	96	96	94
S20 e1	Skara	13th	76	95	85	89
S20 e2	Skara	13th	90	93	96	94
S21 e1	Skara	13th	94	97	89	97
S21 e2	Skara	13th	90	87	97	89
S22 e1	Skara	13th	89	NA	NA	NA
S22 e2	Skara	13th	88	97	96	96
S23 e1	Skara	13th	95	96	91	97
S23 e2	Skara	13th	91	83	96	93
S24 e1	Skara	13th	74	97	93	97
S24 e2	Skara	13th	70	NA	NA	NA
S25 e1	Skara	13th	NA	NA	NA	NA
S25 e2	Skara	13th	89	94	82	90
S26 e1	Skara	13th	91	91	88	91
S26 e2	Skara	13th	94	96	94	95

NOTE.—Each individual was extracted twice (e1 and e2 in the sample column) and each extract was amplified once for each fragment. NA indicates the occasions where we could not get data.

for the dog mitochondrial D-loop. A third, shorter fragment size (93 bp, denoted D93) in the dog D-loop was included to monitor the degradation pattern previously discovered in ancient material (Poinar et al. 2006). The human fragment was identical to the one targeted in the previous study, whereas the dog fragment was not (Malmström et al. 2005). DNA was quantified with real-time PCR following Malmström et al. (2005). Eleven samples were replicated in an independent laboratory (table 1). The long fragments (H148 and D152) were amplified and quantified, and the dog fragment was sequenced.

Additionally, DNA from 34 historic and medieval cattle remains was included in the study and typed with an alternative method to support any results from the dog material. The cattle material was from urban contexts in western Sweden, one sample set was from an 18th century harbor (Marstrand), and yet one from a 13th century town (Skara, table 3). Thus, the cattle material was 10 $\pm$ 5% of the age of the dog material and yet from areas with about the same climate and average year temperature as the dog material. The cattle material was extracted in duplicates (serving as independent observation as there is a large variation in contamination content in duplicate extractions [Malmström et al. 2005]) together with 21 extraction and 10 PCR controls. As we suspected that the material could be well preserved and thus not ideal for contamination studies, we wanted to assure that the proportion of contaminant DNA was sufficiently large for further analyses. Therefore, DNA was extracted according to previous protocols (Yang

et al. 1998; Malmström et al. 2005) but without the additional exposure to HCl and bleach. We did, however, perform a test where we exposed the bone powder from a subset of the samples to 2 different bleach concentrations (0.5 and 3%, table 3). Conserved primers (table 2) were designed to amplify both authentic ancient cattle mtDNA and contaminating human mtDNA for fragments of 4 different sizes (70 bp, 124 bp, 178 bp, and 180 bp) in the 16S rRNA gene. We targeted a substitution (A/T) in nucleotide position 2750 according to accession number V00654 (cattle) and at nt 2952 according to AB055387 (human), where A is specific for cattle DNA and T for human DNA. The substitution was identified and proportionally quantified with pyrosequencing (Ronaghi et al. 1998) using a previously reported protocol (Götherström et al. 2005), with the addition of proportional allele quantification as implemented in the pyrosequencing software (Gruber et al. 2002; Neve et al. 2002).

Deviations between the variance of several of the sample groups of the dog samples rendered the use of parametric statistics problematic. Therefore, our statistical analyses involved the nonparametric Mann–Whitney *U* test. The test provides reasonable power without being dependent upon the shape of the variance. We compared the amount of contamination in the dog samples with previously published data (Malmström et al. 2005). We also compared the contamination yield in the dog bones with the contamination yield in the negative extraction controls in a similar way. For authentication, we compared the proportion of dog DNA compared with human DNA in the replicated samples that yielded data on both species ($n = 7$) with the original data set. To monitor changes, we restricted further statistical testing to samples that yielded quantifiable DNA for both short and long fragments (23 and 14 samples amplified with human or dog-specific primers, respectively, GenBank accession number DQ860843–DQ860864 and AY673648–AY673672 for the previously published data set). We calculated to what extent short fragments exceeded long fragments in the DNA extracts (amount of H148 or D152 fragments/amount of H112 or D111 fragments). The cattle samples were quantified in a different manner to the dog samples and thus yielded a different type of data. We used χ^2 to calculate whether we had identified an excess of samples with an increase in the proportion of authentic aDNA (cattle DNA) compared with contaminating human DNA as the fragment size decreased. This was done for the shortest fragment (70 bp) compared with the increasingly longer fragments (124 bp, 178 bp, and 180 bp). The samples that had yielded results for all 4 fragments were used for a simple regression with size as the independent variable and proportion of aDNA as the dependent variable. As we were interested in the trend, we normalized the data set by dividing all observations in each sample with the one observed for 70 bp prior to calculations. All calculations were performed on STATISTICA 7.

Results and Discussion

Bleach has previously been used on limited amounts of powdered bone and tooth material as a means to decontaminate samples (Kemp and Smith 2005; Salamon et al. 2005). In our data, we found that the level of contamination was significantly lower ($P < 0.001, Z = 7.7924$) among the dog extractions where we treated the powder with bleach than when powdered bone and tooth from the same samples were not treated with bleach (the previously published data set of Malmström et al. [2005]). The amount of authentic aDNA also decreased in these specimens, but to a lesser degree (77% of the authentic aDNA was lost, whereas >99% of the contamination was lost, fig. 2*A*). The cattle material shows a similar effect when exposed to bleach. Furthermore, we noted that with the cattle material, an increase in the bleach concentration from 0.5% to 3% did not appear to enhance the result or provide a higher concentration of authentic aDNA (fig. 3 and table 3). There was significantly more contamination left in the ancient dog extracts than in the extraction and PCR controls ($P = 0.0005, Z = -3.47517$; the controls contained a maximum of 55 molecules and an average of 17 molecules per sample; none contained dog DNA). For the dog-specific primers, the yield ranged from 0 to 14821 (the highest amount was observed in D93) molecules, where the average of all 25 samples was 1491 molecules for D93, 879 molecules for D111, and 188 molecules for D152 (table 4). The contaminating human molecules ranged from 0 to 248 molecules (the highest amount was observed in H148), where the average of all 25 samples was 55 molecules for H112 and 29 molecules for H148 (table 4). The yield appeared to be somewhat higher in the replication (the highest amounts were for D152, which yielded a maximum of 2,034 starting molecules and H148, which yielded a maximum of 1,268 starting molecules). As it was a general trend for contamination as well as for authentic ancient DNA, it

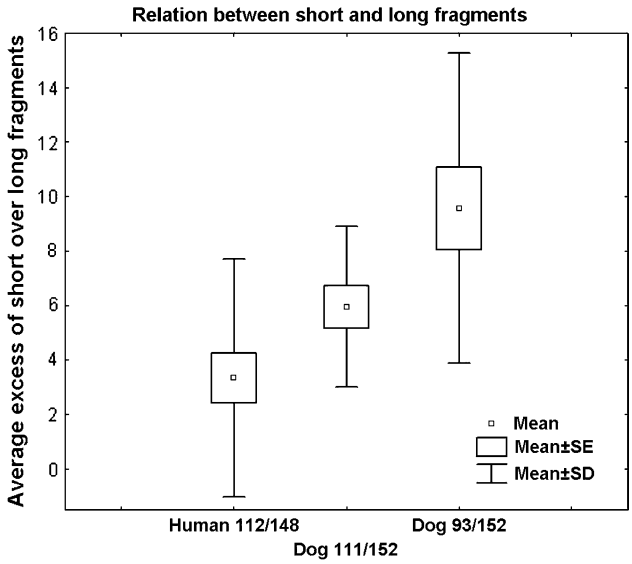

FIG. 1.—Size-related increase of template frequencies. The first box illustrates the relation between the H112 human fragment and the H148 human fragment, where there are 3.4 times more of the shorter than of the longer fragment. The second and third box illustrates the same relation between the D111 and the D152 dog fragment where there is 5.9 times more of the shorter than the longer fragment, and for the D93 and the D152 dog fragment where there is 9.6 times more of the shorter than the longer fragment. The dog observations are based on 14 samples, and the human observations are based on 23 samples.

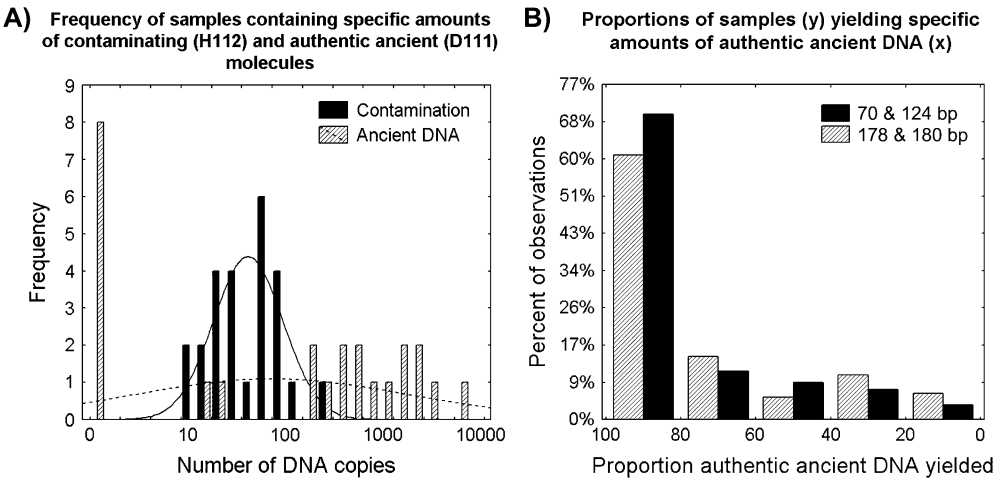

FIG. 2.—Illustration of contamination in ancient material. (*A*) Frequency of dog samples with specific amounts of contaminating (H112) and authentic ancient molecules (D111), $n = 25$. Although the amount of contaminating molecules rarely exceeds 100 and never 300 molecules in the samples exposed to HCl and bleach, the amount of authentic ancient molecules may reach several thousands. (*B*) Frequency (in %) of extractions from cattle samples yielding specific proportion of authentic ancient DNA, $n = 117$ for 70 and 124 bp, and $n = 113$ for 178 and 180 bp. More samples provide short authentic aDNA fragments compared with contaminating DNA fragments, whereas less samples provide long authentic aDNA fragments.

could simply be that the experiment was somewhat more efficient in the replication. However, the proportion between human contamination and ancient dog DNA in the replication did not deviate significantly from the original sample set ($P = 0.28$, $Z = 1.0702$). The increase of DNA yield with decreased fragment size was significantly higher for authentic aDNA than for contaminating human DNA when samples that yielded DNA for long as well as for short fragments were considered ($P = 0.0011$, $Z = -3.2569$, figs. 1 and 2*A*). This trend was further confirmed by quantification in the cattle material (fig. 2*B*, table 4). For the cattle material, the average percent of cattle DNA in the negative controls was 2.3 ± 7.8 (standard deviation [SD]) for 70 bp, 0.3 ± 0.6 (SD) for 124 bp, 0.1 ± 0.6 (SD) for 178 bp, and 0 ± 0 (SD) for 180 bp. Only in 1 case out of 84 did we register a sufficiently high proportion of cattle DNA (35% in one of the 70 bp amplifications from a negative extraction control) to conclude with certainty that there actually was cattle DNA in the negative control (Gruber et al. 2002; Neve et al. 2002). A significant difference for more authentic aDNA in the shorter fragments than in the longer fragments was obtained in 2 cases out of 3, when longer fragments were compared with the 70 bp fragment. This pattern was evident when the size difference increased (70 bp/124 bp: $P = 0.25$, $\chi^2 = 1.35$, $n = 54$; 70 bp/178 bp: $P = 0.037$, $\chi^2 = 4.36$, $n = 48$; 70 bp/180 bp: $P = 0.024$, $\chi^2 = 5.06$, $n = 59$; and 1 degree of freedom in all cases). A simple regression also indicated a significant correlation for fragment size and proportion of contamination ($P = 0.0035$, $F = 8.736$). However, this difference is not evident when fragments are visualized on agarose gels after conventional PCR. All of the 25 dog samples showed presence of human-specific amplicons for the H112 fragment and 23 of them did so also for the H148 fragment, whereas 17 showed dog-specific amplicons for the D111 fragment and 15 did so for the D152 fragment.

Aggressive pretreatment of the dog material, in our case with HCl and bleach, eliminated a large proportion of the contaminant DNA (fig. 2*A* and 3). We also conclude that authentic aDNA will show a more rapid increase in yield with decreased fragment size than contaminating DNA, even when using material excavated a century ago, which has been well handled since and thus likely contains a large number of old contaminant DNA molecules. We could detect this pattern with 2 different quantification methods and in 2 different types of data sets. This asymmetrical behavior is the only known detectable and quantifiable difference between contaminating modern human and ancient human DNA, and we therefore suggest that it can be used to support claims for authentic ancient human DNA.

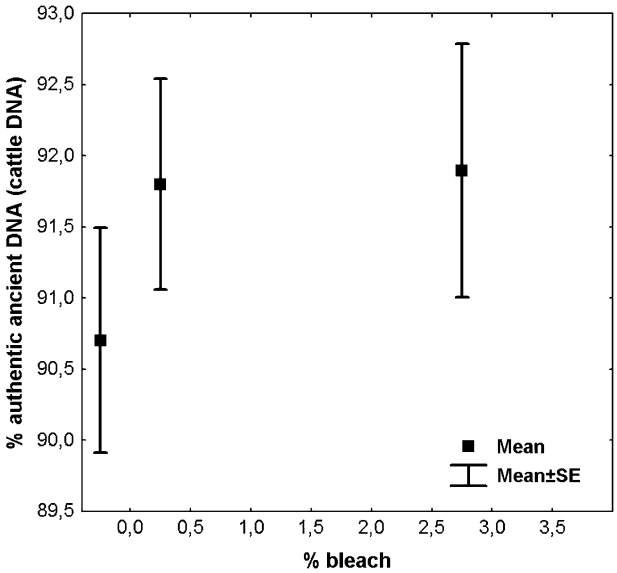

FIG. 3.—Cattle remains were treated with bleach in different concentrations (0%, $n = 64$; 0.5%, $n = 77$; and 3%, $n = 68$) prior to pyrosequencing. The proportion of contamination appears to decrease with bleach treatment, but whether the treatment is with 0.5% or 3% bleach appears to have little effect on the final result.

Table 4
Average and Maximum Values for Numbers of Dog and Human Mitochondrial Fragments in Dog Samples and Proportion of Human Fragments in Cattle Samples

	Max	Mean
Dog (n = 25)		
D93	14821	1491
D111	7817	879
D152	1904	188
Ratio 93/152	7.78	7.93
Ratio111/152	4.2	4.68
Human (n = 25)		
H112	261	55
H148	248	29
Ratio 112/148	1.05	1.9
% Human in Cattle		
70 (n = 61)	80	20
124(n = 56)	98	19
178 (n = 51)	100	23
180 (n = 62)	89	25

NOTE.—n is based on number of extractions.

Typically, a human aDNA extract yields several different haplotypes, both authentic and contaminant. However, when an internal shorter type-specific fragment is targeted, the number of ancient haplotype copies should increase disproportionately compared with the contaminant haplotypes. Quantification of haplotypes or alleles in amplicons of different fragment lengths should thus allow researchers to single out authentic human DNA.

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Literature Cited

Bandelt HJ. 2005. Mosaics of ancient mitochondrial DNA: positive indicators of nonauthenticity. Eur J Hum Genet. 13:1106–1112.

Bouwman AS, Chilvers ER, Brown KA, Brown TA. 2006. Brief communication: identification of the authentic ancient DNA sequence in a human bone contaminated with modern DNA. Am J Phys Anthropol. 131:428–431.

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

Dalton R. 2005. Caveman DNA hints at map of migration. Nature. 436:162.

Gilbert MTP, Rudbeck L, Willerslev E, Hansen AJ, Smith C, Penkman KEH, Prangenberg K, Nielsen-Marsh CM, Jans ME, Arthur P. 2005. Biochemical and physical correlates of DNA contamination in archaeological human bones and teeth excavated at Matera, Italy. J Archaeol Sci. 32:785–793.

Götherström A, Anderung C, Hellborg L, Elburg R, Smith C, Bradley DG, Ellegren H. 2005. Cattle domestication in the Near East was followed by hybridization with aurochs bulls in Europe. Proc Biol Sci. 272:2345–2350.

Gruber JD, Colligan PB, Wolford JK. 2002. Estimation of single nucleotide polymorphism allele frequency in DNA pools by using pyrosequencing. Hum Genet. 110:395–401.

Haak W, Forster P, Bramanti B, Matsumura S, Brandt G, Tanzer M, Villems R, Renfrew C, Gronenborn D, Alt KW, Burger J. 2005. Ancient DNA from the first European farmers in 7500-year-old Neolithic sites. Science. 310:1016–1018.

Handt O, Hoss M, Krings M, Pääbo S. 1994. Ancient DNA: methodological challenges. Experientia. 50:524–529.

Handt O, Krings M, Ward RH, Pääbo S. 1996. The retrieval of ancient human DNA sequences. Am J Hum Genet. 59:368–376.

Hofreiter M, Serre D, Poinar HN, Kuch M, Paabo S. 2001. Ancient DNA. Nat Rev Genet. 2:353–359.

Kemp BM, Smith DG. 2005. Use of bleach to eliminate contaminating DNA from the surface of bones and teeth. Forensic Sci Int. 154:53–61.

Malmström H, Storå J, Dalén L, Holmlund G, Götherström A. 2005. Extensive human DNA contamination in extracts from ancient dog bones and teeth. Mol Biol Evol. 22:2040–2047.

Neve B, Froguel P, Corset L, Vaillant E, Vatin V, Boutin P. 2002. Rapid SNP allele frequency determination in genomic DNA pools by pyrosequencing. Biotechniques. 32:1138–1142.

Noonan JP, Hofreiter M, Smith D, Priest JR, Rohland N, Rabeder G, Krause J, Detter JC, Pääbo S, Rubin EM. 2005. Genomic sequencing of Pleistocene cave bears. Science. 309:597–599.

Poinar HN, Schwarz C, Qi J, et al. (13 co-authors). 2006. Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA. Science. 311:392–394.

Richards MB, Sykes BC, Hedges REM. 1995. Authenticating DNA extracted from ancient skeletal remains. J Archaeol Sci. 22:291–299.

Ronaghi M, Uhlén M, Nyrén P. 1998. A sequencing method based on real-time pyrophosphate. Science. 281:363, 365.

Salamon M, Tuross N, Arensburg B, Weiner S. 2005. Relatively well preserved DNA is present in the crystal aggregates of fossil bones. Proc Natl Acad Sci USA. 102:13783–13788.

Sampietro ML, Caramelli D, Lao O, Calafell F, Comas D, Lari M, Agusti B, Bertranpetit J, Lalueza-Fox C. 2005. The genetics of the pre-Roman Iberian Peninsula: a mtDNA study of ancient Iberians. Ann Hum Genet. 69:535–548.

Sampietro ML, Gilbert MTP, Lao O, Caramelli D, Lari M, Bertranpetit J, Lalueza-Fox C. 2006. Tracking down human contamination in ancient human teeth. Mol Biol Evol. 23:1801–1807.

Smith CI, Chamberlain AT, Riley MS, Cooper A, Stringer CB, Collins MJ. 2001. Neanderthal DNA. Not just old but old and cold? Nature. 410:771–772.

Yang DY, Eng B, Waye JS, Dudar JC, Saunders SR. 1998. Technical note: improved DNA extraction from ancient bones using silica-based spin columns. Am J Phys Anthropol. 105:539–543.

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