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A Minimally Destructive Protocol for DNA Extraction from Ancient Teeth

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Running Title: Minimally Destructive DNA Extraction from Ancient Teeth
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1 ABSTRACT

2 Ancient DNA sampling methods—although optimized for efficient DNA extraction—are

3 destructive, relying on drilling or cutting and powdering (parts of) bones and teeth. As the

4 field of ancient DNA has grown, so have concerns about the impact of destructive

5 sampling of the skeletal remains from which ancient DNA is obtained. Due to a particularly

6 high concentration of endogenous DNA, the cementum of tooth roots is often targeted for

7 ancient DNA sampling, but standard destructive sampling methods often result in the loss

8 of at least one entire root. Here, we present a minimally destructive method for extracting

9 ancient DNA from dental cementum present on the surface of tooth roots. This method

10 does not require destructive drilling or grinding, and, following extraction, the tooth

11 remains safe to handle and suitable for most morphological studies, as well as other

12 biochemical studies, such as radiocarbon dating. We extracted and sequenced ancient

13 DNA from 30 teeth (and 9 corresponding petrous bones) using this minimally destructive

14 extraction method in addition to a typical tooth sampling method. We find that the

15 minimally destructive method can provide ancient DNA that is of comparable quality to

16 extracts produced from teeth that have undergone destructive sampling processes.

17 Further, we find that a rigorous cleaning of the tooth surface combining diluted bleach

18 and UV light irradiation seems sufficient to minimize external contaminants usually

19 removed through the physical removal of a superficial layer when sampling through

20 regular powdering methods.

INTRODUCTION

Over the past decade, the field of ancient DNA has experienced a rapid increase in the number of ancient genomes published each year (Slatkin and Racimo 2016) as a consequence of advances in ancient DNA sampling (Gamba et al. 2014; Damgaard et al. 2015), extraction (Dabney et al. 2013a; Rohland et al. 2018), and enrichment (Carpenter et al. 2013; Fu et al. 2013) techniques. As our ability to sequence large numbers of ancient individuals has increased, discussions about the destructive nature of ancient DNA sampling—which typically requires drilling or cutting and powdering ancient bones and teeth—have become more prominent (Makarewicz et al. 2017; Prendergast and Sawchuk 2018; Sirak and Sedig 2019). The identification of the osseous inner ear, and specifically the cochlea (located in the petrous portion of the temporal bone), as an optimal source of ancient DNA (Gamba et al. 2014; Pinhasi et al. 2015; Pinhasi et al. 2019) is one of the driving factors in this revolution, making it possible to access ancient DNA from geographic regions with climatic conditions unfavorable to ancient DNA preservation. However, accessing this optimal source of ancient DNA results in the destruction of the inner ear morphology, which is a valuable source of morphological information (de León et al. 2018). While there are protocols that reduce the destructive nature of sampling, by sampling from the ossicles of the inner ear (Sirak et al. 2020) or performing targeted drilling of the cochlea through the cranial base of complete or reconstructed crania (Sirak et al. 2017), some destruction (including that of morphologically-informative inner ear components) is inevitable. As a consequence, this and other less-invasive methods may be considered unsuitable in cases where samples are of particular anthropological value and are subject to stringent restrictions on permissible sampling practices.

44

45   Teeth are a valuable alternative to the sampling of the cochlea (Gamba et al. 2014;

46   Damgaard et al. 2015), especially because they are particularly numerous in osteological

47   collections, due to the fact that individuals have many more teeth than petrous bones and

48   to their resistance to taphonomic decomposition. Despite this, little has been published

49   outlining optimal practices for sampling from teeth. Traditionally, the standard practice

50   has been to grind or drill large chunks of the tooth root to a powder (Rohland and Hofreiter

51   2007), as the crown enamel is largely inorganic and is therefore unlikely to contain a

52   substantial amount of endogenous DNA (Higgins and Austin 2013). In an attempt to

53   minimize potential external contaminants, the surface layer is often removed to access

54   the "untouched" dentine and pulp. However, this practice removes some, if not all, of the

55   thin layer of cementum that coats the inferior portion of dental roots.

56

57   The cellular cementum is rich in cementocytes, which are DNA containing cells that

58   remain encased in the mineral structure of the tooth after death (Bosshardt and Selvig

59   1997). Cementum also shares several histological properties with the cochlear region of

60   the petrous that are thought to contribute to its high level of DNA preservation, including

61   similarities between cementocytes (Zhao et al. 2016) and osteocytes, which are

62   hypothesized to be serve as repositories of ancient DNA in bones (Bell et al. 2008; Pinhasi

63   et al. 2015). Like the cochlea, cementum also does not undergo remodeling (but, unlike

64   the cochlea, it continues to accumulate throughout life) and the haphazard organization

65   of collagen fibers in cementum resembles that of woven bone (Freeman 1994; Grzesik et

66   al. 2000). Assessment of DNA preservation in ancient teeth shows that dental cementum

67   contains a substantially higher proportion of endogenous DNA than dentine from the

68   same tooth (Damgaard et al. 2015). Furthermore, in a direct comparison between

69   cementum and petrous samples, Hansen et al. (2017) find that cementum and petrous

70   yield a comparable amount of endogenous DNA in well-preserved samples, although in

71   poorly-preserved individuals, the petrous yields a higher proportion of endogenous

72   molecules. The only published method for sampling DNA from the cementum

73   recommends a targeted method for extracting DNA from teeth using an "inside-out"

74   approach that involves removing the crown and subsequently using a fine drill to remove

75   as much pulp and dentine as possible from the tooth root to ultimately obtain a "case" of

76   cementum (Damgaard et al. 2015). However, this valuable approach may still not be able

77   to perfectly isolate the extremely thin and brittle layer of cementum, which ranges from

78   20-50 μm thick at the cementoenamel junction, to 150-200 μm thick at the apex of the

79   root (Freeman 1994).

80

81   Here, we present an alternative, minimally destructive protocol for sampling ancient DNA

82   from tooth cementum that does not require drilling or cutting, thereby maintaining the

83   morphological integrity of the tooth. The technique isolates ancient DNA from the

84   cementum of tooth roots by directly exposing the outermost layer of a portion of the tooth

85   root to a lysis buffer for a short incubation period, following a non-destructive

86   decontamination procedure. Similar less destructive methods have been reported in

87   previous PCR-based mitochondrial ancient DNA studies (Rohland et al. 2004; Bolnick et

88   al. 2012) and in forensic contexts (Correa et al. 2019). However, the ancient DNA

89   obtained using these strategies was typically less well preserved and of a lesser quantity

90   than DNA obtained using more destructive methods. Additionally, in some cases

91   (Rohland et al. 2004), the hazardous chemicals used during sampling may have

92   compromised safe handling and future chemical analyses of the remains. In this study,

93   we conduct a systematic evaluation of the application of a minimally destructive sampling

94   technique in a next generation sequencing context. This protocol is further optimized by

95   enabling targeted sampling from the very thin dental cementum layer, which increases

96   the quality of ancient DNA sampled from the tooth while giving researchers the ability to

97   fully preserve the dental crowns and all but the fine external detail of the roots. After

98   sampling, teeth can be safely handled and remain suitable for subsequent morphological

99   and biomolecular analyses, such as radiocarbon dating (Korlević et al. 2018).

RESULTS

101   We selected thirty ancient individuals (Table 1; Supplementary Table 1) for a comparative

102   analysis of the quality of ancient DNA—as measured through metrics such as the

103   proportion of endogenous molecules of shotgun data, sample complexity and

104   contamination rate—that could be obtained from an individual using this minimally

105   destructive extraction method versus standard sampling procedures that rely on cutting

106   and powdering tooth samples. From each individual we sampled a single multi-rooted

107   tooth, from which the roots were removed via cutting (note that the tooth roots were cut

108   in order to make it possible to process the samples using several independent methods,

109   but cutting is not required by the minimally destructive sampling protocol) and were each

110   randomly assigned to undergo one of the following extraction treatments. We extracted

111   ancient DNA from a tooth root that was processed using the minimally destructive

112   extraction protocol described in this paper (Method "MDE"; for "Minimally Destructive

113   Extraction") and a second whole tooth root of the same tooth, that was completely

114   powdered via milling (Method "WTR"; for "Whole Tooth Root"). We also generated

115   extracts from powder produced from petrous bones for 10 of the same individuals using

116   the method described by Pinhasi et al. (2019) (Method "P"; for "Petrous"). In one case

117   (individual 3), we discovered through subsequent bioinformatic analyses that the petrous

118   bone and tooth sampled did not originate from the same individual, and we therefore

119   exclude the petrous bone results from further analyses. DNA preservation in two

120   individuals (5 and 6) was uniformly poor, with no more than 10,000 sequences aligning

121   to the 1.24 million sites captured through targeted enrichment (out of ~5 million unique

122   reads sequenced) from any of the libraries generated. Furthermore, all of these double-

123   stranded libraries exhibited C-to-T damage rates at the terminal ends of molecules of less

124   than 3%—the recommended minimum threshold for assessing ancient DNA authenticity

125   in partially UDG treated libraries (Rohland et al. 2015). These samples are considered to

126   have 'failed' screening for authentic ancient DNA and are not included in the statistical

127   analyses. Additionally, individual 22 yielded relatively poor results for both treatments.

128   Only 533 reads (out of ~4 million unique reads sequenced) aligned to the 1.24 million

129   sites targeted in the nuclear genome for the MDE treatment, making it impossible to

130   calculate several of the reported metrics. While we did obtain enough reads (23,239 reads

131   out of ~18 million unique reads sequenced) for some analyses to produce results for the

132   tooth root that underwent Method WTR, the relatively low rate of mitochondrial match to

133   the consensus (0.860) suggests that this sample is likely contaminated. Based on these

134   results, we also chose to exclude individual 22 from statistical analyses. However, we

135 note that there are no significant changes to the reported statistics when the excluded

136 individuals are included in calculations for which metrics from both treatments are

137 available (Supplementary Table 2). For all statistical calculations, we included data from

138 all other samples, which were processed as either double-stranded (samples 1-10) or

139 single-stranded (samples 11-30) libraries. Results where each of these methods were

140 analyzed separately are reported in Supplementary Table 2.

Individual	Method Applied/Element Type*	Sample Origin and Age (Years Before Present)	Percent Endogenous (pre-capture libraries)	Number of sequences aligning to the 1240k targeted nuclear sites (captured libraries)	Coverage on 1240k autosomal targets (captured libraries)	Median length of sequences aligning to the human genome (pre-capture libraries)	C-to-T damage rate at 5' end of molecules aligning to the human genome (pre-capture libraries)	Complexity (Percentage of unique reads out of 1,000,000 sequenced reads) (captured libraries)	Complexity (Informative Sequence Content)	Rate of mitochondrial match to the consensus (95% confidence interval) (captured libraries) ***	Autosomal Contamination Rate (contamLD) (captured libraries)****	Contamination Rate (Assessed in Genetic Males via ANGSD) (captured libraries)****
1	P	Urziceni, Romania 6,300-6,050 BP	68.23%	732856	2.94	44	0.111	92.10%	1.72E+10	0.992 +/- 0.006	-0.003 +/- 0.005	0.007
	MDE		34.22%	422192	0.37	48	0.072	30.50%	1.12E+09	0.990 +/- 0.008	-0.008 +/- 0.023	0.025
	WTR		12.50%	648760	0.88	46	0.055	51.80%	8.74E+09	0.997 +/- 0.004	0.008 +/- 0.011	0.006
2	P	Urziceni, Romania 6,300-6,050 BP	23.19%	713660	2.78	44	0.107	91.20%	2.93E+11	0.986 +/- 0.010	-0.01 +/- 0.006	0.006
	MDE		8.51%	507713	0.51	46	0.055	38.10%	2.74E+09	0.976 +/- 0.012	-0.033 +/- 0.023	0.003
	WTR		1.19%	8438	3.32	50	0.045	0.90%	2.03E+06	0.984 +/- 0.010	-0.097 +/- 0.077	..
3**	MDE	Glăvăneşti, Romania 5,450-3,050 BP	2.01%	29234	0.02	39	0.102	2.80%	1.24E+07	0.803 +/- 0.087	..	..
	WTR		0.65%	62005	0.04	39	0.128	6.30%	4.60E+08	0.946 +/- 0.029	..	..
4	P	Glăvăneşti, Romania 5,450-3,050 BP	1.65%	165145	0.12	39	0.147	14.30%	9.78E+08	0.936 +/- 0.024	..	..
	MDE		72.77%	624069	0.91	47	0.055	53.00%	1.52E+10	0.979 +/- 0.011	-0.010 +/- 0.014	0.010
	WTR		19.69%	633735	0.87	47	0.047	52.30%	5.73E+09	0.993 +/- 0.005	0.000 +/- 0.017	0.002
5**	P	Ras al Hamra, Oman 5,650-5,150 BP	0.10%	6226	0	60	0.000	..	2.90E+06	..	..	..
	MDE		2.74%	8364	0.01	59	0.000	0.90%	2.00E+06	..	..	..
	WTR		1.48%	6655	0	61	0.000	0.70%	2.15E+06	..	..	..
6**	P	Ras al Hamra, Oman 5,650-5,150 BP	0.17%	6530	0	58	0.000	0.70%	3.61E+06	..	..	..
	MDE		0.97%	8860	0.01	58	0.019	1.00%	1.61E+06	..	..	..
	WTR		0.24%	7846	0.01	58	0.029	0.90%	2.84E+06	..	..	..
7	P	Cimișlia, Rep. of Moldova 2,050-1,850 BP	2.74%	185208	0.15	38	0.282	16.40%	6.36E+09	0.988 +/- 0.009	0.004 +/- 0.018	..
	MDE		57.34%	486828	0.49	44	0.135	34.50%	3.81E+09	0.983 +/- 0.009	0.022 +/- 0.009	..
	WTR		8.34%	530939	0.58	45	0.064	39.80%	4.50E+09	0.993 +/- 0.006	-0.013 +/- 0.011	..
8	P	Ciumai, Rep. of Moldova 4,000-1,000 BP	51.70%	712417	2.76	44	0.149	90.20%	2.73E+11	0.994 +/- 0.005	-0.009 +/- 0.003	0.004
	MDE		0.74%	223292	0.17	45	0.077	19.00%	3.92E+08	0.997 +/- 0.003	-0.002 +/- 0.023	..
	WTR		31.64%	683354	2.54	42	0.147	87.40%	1.39E+11	0.954 +/- 0.014	-0.01 +/- 0.005	0.007
9	P		43.68%	716356	2.53	45	0.130	86.70%	1.73E+11	0.989 +/- 0.007	-0.003 +/- 0.006	..

#	Type	Location										
	MDE	Polgár-Ferenci-hát, Hungary 7,280-7,035 BP	0.69%	33735	0.02	44	0.163	3.40%	1.20E+07	0.936 +/- 0.028	..	..
	WTR		6.81%	335725	0.32	39	0.196	27.10%	2.22E+09	0.987 +/- 0.007	0.011 +/- 0.011	..
10	P	Polgár-Ferenci-hát, Hungary 7,280-7,035 BP	35.59%	726484	2.67	45	0.124	88.60%	1.88E+11	0.992 +/- 0.006	-0.002 +/- 0.006	0.008
	MDE		38.90%	654431	0.92	50	0.080	53.70%	8.43E+09	0.988 +/- 0.008	-0.008 +/- 0.011	0.008
	WTR		1.92%	425428	0.41	47	0.068	33.70%	3.32E+09	0.990 +/- 0.007	-0.001 +/- 0.006	0.009
11	MDE	Kesznyéten-Szérűskert, Hungary 2,600-2,400 BP	44.35%	501547	0.57	51	0.122	34.30%	5.09E+09	0.985 +/- 0.008	0.013 +/- 0.019	..
	WTR		24.54%	454947	0.5	50	0.135	31.20%	2.63E+09	0.983 +/- 0.008	-0.008 +/- 0.022	..
12	MDE	Kesznyéten-Szérűskert, Hungary 2,600-2,400 BP	38.54%	203330	0.19	45	0.187	14.00%	1.83E+09	0.972 +/- 0.019	-0.059 +/- 0.03	..
	WTR		4.64%	223768	0.21	46	0.176	15.60%	3.15E+09	0.994 +/- 0.005	-0.083 +/- 0.044	..
13	MDE	Kesznyéten-Szérűskert, Hungary 2,600-2,400 BP	25.12%	478265	0.56	41	0.177	32.40%	1.58E+09	0.995 +/- 0.004	0.015 +/- 0.017	0.009
	WTR		2.52%	56389	0.05	46	0.190	4.70%	7.08E+08	0.994 +/- 0.005	..	..
14	MDE	Kesznyéten-Szérűskert, Hungary 2,600-2,400 BP	69.20%	645264	0.91	45	0.145	45.80%	1.48E+10	0.983 +/- 0.010	0.006 +/- 0.014	..
	WTR		29.45%	487615	0.55	48	0.144	32.80%	5.61E+09	0.982 +/- 0.010	-0.016 +/- 0.021	..
15	MDE	Kesznyéten-Szérűskert, Hungary 2,600-2,400 BP	4.02%	9275	0.01	50	0.190	1.00%	3.43E+08	0.845 +/- 0.076	..	..
	WTR		2.16%	2523	0	62	0.144	0.00%	4.28E+08	0.886 +/- 0.075	..	..
16	MDE	Mezőkeresztes-Cethalom, Hungary 2,770-2.494 BP	6.30%	48824	0.04	45	0.225	4.00%	6.69E+08	0.954 +/- 0.022	..	..
	WTR		1.55%	5369	0.01	59	0.162	0.00%	1.95E+09	0.983 +/- 0.014	..	..
17	MDE	Hajdúdorog-Szállásföld, Hungary 3,700-2,800 BP	54.62%	213244	0.2	51	0.148	15.10%	1.99E+09	0.978 +/- 0.011	0.026 +/- 0.038	..
	WTR		25.66%	443564	0.55	37	0.299	33.20%	1.69E+10	0.992 +/- 0.006	0.003 +/- 0.011	..
18	MDE	Polgár Kenderföld, Hungary 4,300-3,600 BP	12.27%	198726	0.19	44	0.184	14.20%	1.50E+09	0.987 +/- 0.009	-0.045 +/- 0.033	..
	WTR		37.08%	409177	0.43	47	0.181	26.60%	4.55E+09	0.991 +/- 0.006	0.011 +/- 0.021	0.012
19	MDE	Köröm-Kápolnadomb, Hungary 3,700-2,800 BP	25.75%	116478	0.11	46	0.161	8.80%	8.29E+08	0.974 +/- 0.016	..	..
	WTR		1.16%	83929	0.07	48	0.179	6.80%	9.45E+08	0.985 +/- 0.008	..	..
20	MDE	Besenyszög Berek-ér partja, Hungary 2,250-2,150 BP	63.90%	230522	0.22	55	0.081	16.30%	1.29E+09	0.989 +/- 0.008	0.062 +/- 0.015	..
	WTR		59.50%	500669	0.66	39	0.155	37.20%	1.58E+09	0.971 +/- 0.015	-0.029 +/- 0.015	..
21	MDE	Dereivka, Ukraine 8,392-7,927 BP	71.06%	750807	1.2	45	0.236	51.90%	1.41E+09	0.959 +/- 0.014	-0.011 +/- 0.009	..
	WTR		3.90%	150735	0.14	44	0.229	11.00%	2.21E+09	0.978 +/- 0.011	..	..
22	MDE	Dereivka, Ukraine 7,500-6,800 BP	0.42%	533	0	42	0.152	0.00%	..	..	..	..
	WTR		1.38%	23239	0.02	37	0.291	2.10%	4.07E+08	0.860 +/- 0.062	..	..
23	MDE	Ekven, Russia 1,400-900 BP	51.31%	615980	0.83	51	0.045	44.40%	9.50E+08	0.987 +/- 0.009	0.034 +/- 0.012	0.000
	WTR		16.37%	314638	0.32	44	0.103	22.40%	3.99E+09	0.990 +/- 0.008	-0.048 +/- 0.033	0.008
24	MDE	Ekven, Russia 1,030-790 BP	3.44%	306064	0.31	52	0.062	22.20%	3.38E+09	0.983 +/- 0.008	-0.03 +/- 0.046	0.007
	WTR		26.22%	636393	0.85	56	0.043	44.20%	7.79E+09	0.979 +/- 0.008	0.071 +/- 0.015	0.003
25	MDE	Ekven, Russia 1,380-1,010 BP	36.82%	284448	0.28	49	0.044	19.80%	1.65E+08	0.992 +/- 0.005	0.006 +/- 0.018	..
	WTR		65.86%	821749	1.57	53	0.051	62.80%	2.04E+09	0.989 +/- 0.006	-0.03 +/- 0.017	0.001
26	MDE	Uelen, Russia 1,100-750 BP	34.43%	496909	0.59	45	0.049	34.60%	9.06E+08	0.991 +/- 0.005	0.038 +/- 0.029	..
	WTR		1.38%	236589	0.23	52	0.071	17.70%	2.41E+09	0.997 +/- 0.003	0.109 +/- 0.049	..
27	MDE	Ekven, Russia 1,310-930 BP	59.86%	470046	0.52	50	0.039	31.10%	3.98E+08	0.995 +/- 0.005	0.004 +/- 0.034	0.008
	WTR		48.05%	486910	0.55	50	0.082	33.10%	5.27E+09	0.998 +/- 0.003	-0.022 +/- 0.031	0.006
28	MDE	Ekven, Russia 6,350-6,260 BP	62.18%	288212	0.28	50	0.107	19.40%	2.17E+09	0.995 +/- 0.004	-0.047 +/- 0.036	-0.001
	WTR		15.19%	238870	0.23	49	0.092	17.20%	1.33E+09	0.998 +/- 0.002	0.061 +/- 0.043	..
29	MDE	Ust Belaya, Russia 4,840-4,490 BP	26.08%	359586	0.37	43	0.065	24.40%	5.41E+08	0.990 +/- 0.006	-0.01 +/- 0.027	-0.002
	WTR		18.30%	241115	0.23	50	0.071	17.60%	3.39E+08	0.991 +/- 0.005	-0.069 +/- 0.026	..
30	MDE	Volosovo-Danilovo, Russia 4,000-2,000 BP	62.43%	220045	0.21	46	0.080	15.40%	1.60E+09	0.992 +/- 0.008	-0.032 +/- 0.045	..
	WTR		40.05%	448058	0.49	48	0.119	29.90%	6.04E+09	0.997 +/- 0.003	0.003 +/- 0.013	0.015

141 **Table 1. Sample Information** All estimates are made based on data produced from libraries that underwent
142 the 1240k capture unless otherwise specified.
143 *Sampling/Extraction Methods: P- Powdered Petrous Bone (Pinhasi et al. 2019), standard extraction
144 (Dabney et al. 2013a); MDE- Tooth Root processed via Minimally Destructive Extraction; WTR- Whole
145 Tooth Root, powdered with standard extraction (Dabney et al. 2013a). Extracts for individuals 1-10 were
146 processed entirely manually and underwent partial UDG treatment followed by double stranded library
147 preparation, while extracts for individuals 11-30 were processed robotically following incubation in
148 extraction buffer (Rohland et al 2019, buffer D) and processed using USER treatment followed by single
149 stranded library preparation.
150 ** Note that sample 3P was excluded from comparisons as it was determined bioinformatically that the
151 petrous bone and tooth sampled did not originate from the same individual. Note also that the DNA
152 preservation in samples 5 and 6 was too poor for further analysis.
153 *** Contamination estimates are not reported for samples which did not produce sufficient quality data to
154 generate a contamination estimate based on either mitochondrial, autosomal or X-chromosome data. For
155 X-chromosome based contamination estimates, ANGSD can only estimate contamination rates for
156 individuals determined to be genetically male. Individuals who are female or for whom sex cannot be
157 determined (sex ND) are noted.

158 ## Physical Impact of Minimally Destructive Extraction Protocol

159 We photographed each tooth root processed using the minimally destructive extraction

160 protocol immediately prior to extraction and 24 hours after extraction to allow for the

161 complete drying of the roots (Figure 1; Supplementary Figure 1). A slight degradation of

162 the outer tooth root surface is visible for many of the samples, as the portion of the tooth

163 root exposed to extraction buffer shows a visible change in color and/or diameter relative

164 to the unexposed portion. In the case of two of the most poorly preserved samples

165 (individuals 5 and 6), the tooth roots—one of which broke in two when cut from the tooth

166 crown—crumbled during removal of the parafilm that covered the tops of the roots after

167 the incubation in extraction buffer. These results suggest that users should exercise

168 caution when applying this method to very friable teeth that are already susceptible to

169 crumbling or being crushed.
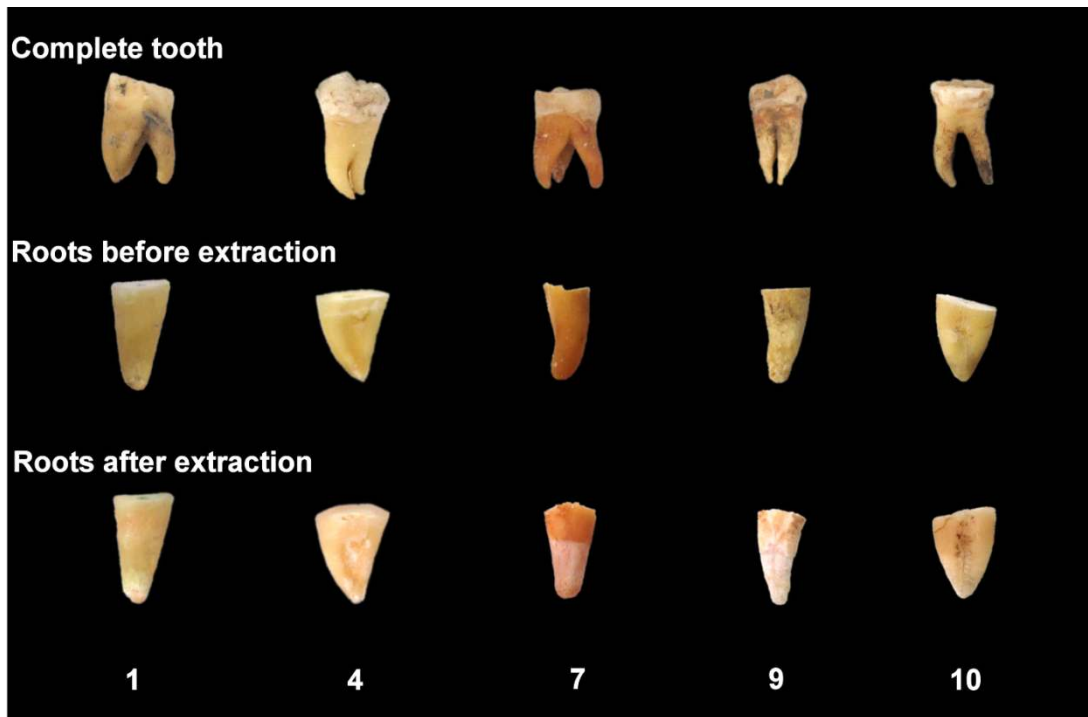
Figure 1. Tooth roots before and after minimally destructive extraction. The complete tooth is shown prior to processing (top). Tooth roots are shown immediately prior to extraction (middle) and 24 hours after extraction (bottom). See Supplementary Figure 1 for before and after images of all sampled teeth.

Comparison of Minimally Destructive Extraction Protocol versus Powder-Based Extraction Protocols

Following bioinformatic processing, we generated summary statistics for each extract, including metrics of sample complexity and contamination rates (Table 1, Supplementary Table 1). In the following section, for each individual we compare the quality of ancient DNA yielded by the minimally destructive extraction method (Method MDE) to that produced by the destructive, traditional sampling methods (Methods WTR and P), using a Wilcoxon signed-rank test. The null hypothesis is that the difference between pairs of data generated using Method MDE and Method WTR or P follows a symmetric distribution around zero. The alternative hypothesis is that the difference between the paired data does not follow a symmetric distribution around zero. A threshold of p-value=0.05 is used

186   to denote significance which can only be achieved if there are a minimum of 6

187   comparisons per test.

Extraction Efficiency

189   In order to assess the efficiency of the minimally destructive extraction method, we first

190   compare the proportion of endogenous molecules (i.e. molecules that align to the human

191   reference genome, hg19) in samples produced using each extraction method and

192   sequenced via shotgun (i.e. pre-capture) sequencing. While we observe a high degree of

193   variability (Figure 2a; Table 1) between treatment types for each individual, there is a

194   statistically significant difference in the proportion of endogenous molecules sequenced

195   using the MDE and WTR methods (p-value=0.004), with an average of 35.8% and 18.8%

196   endogenous molecules for each extraction method, respectively. These results support

197   previous assertions that the outer cementum layer of the tooth root, which is targeted by

198   the MDE method, contains a higher proportion of endogenous molecules than other

199   portions of the tooth root (Damgaard et al. 2015). In contrast, we do not observe a

200   significant difference in the proportion of endogenous molecules between methods MDE

201   and P (p-value=1.000) (Supplementary Figure 2a), with an average of 36.4% endogenous

202   observed when sampling from the petrous. These results are again consistent with claims

203   that the petrous and tooth cementum both contain relatively high proportions of

204   endogenous molecules (Damgaard et al. 2015; Hansen et al. 2017). While the high

205   proportion of endogenous molecules obtained using the MDE method is promising,

206   measuring the fraction of endogenous molecules in a sample does not tell us about the
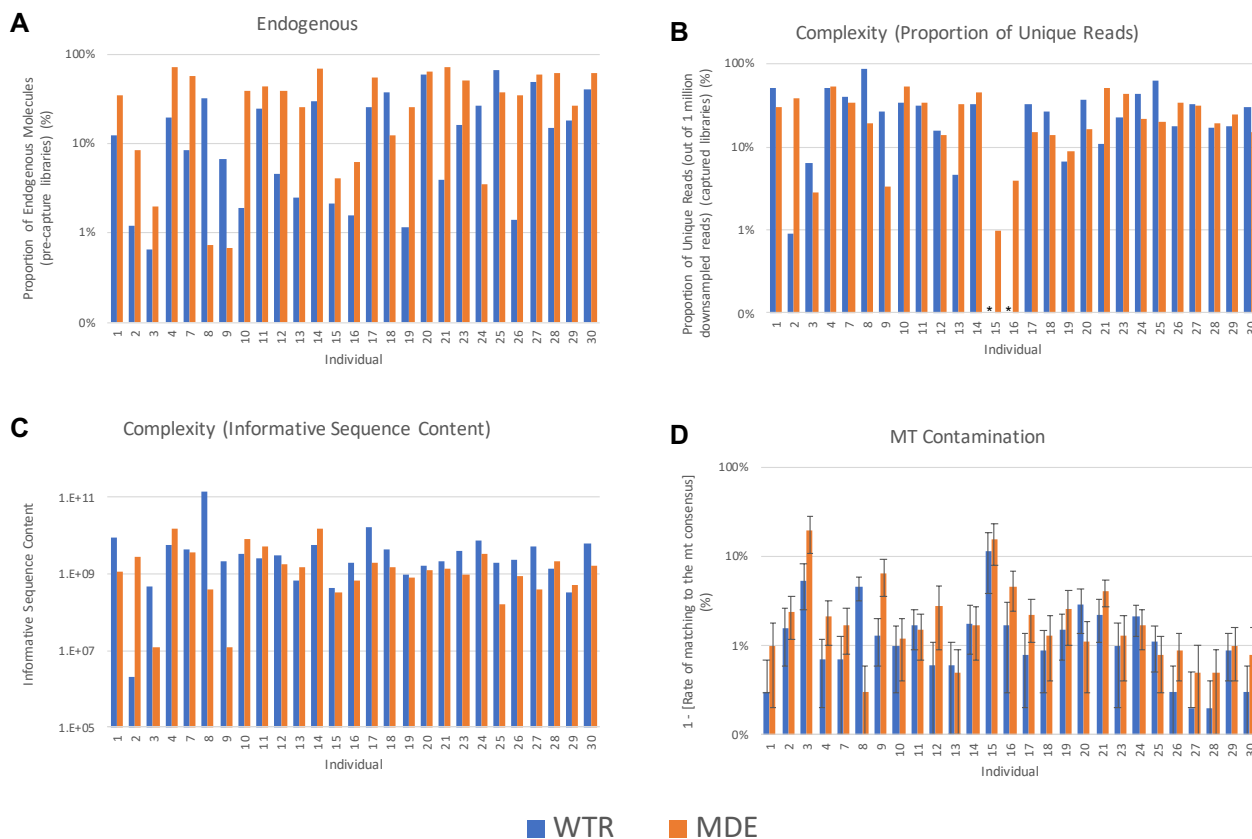
207   total amount of DNA obtained using each method.

Figure 2: Sample Quality A comparison of the quality of data produced by WTR (Whole Tooth Root) and MDE (Minimally Destructive Extraction) Methods in samples that passed quality filtering. (A) The proportion of endogenous molecules in data obtained via shotgun sequencing. (B) The complexity of each sample, as measured by the proportion of unique reads out of 1,000,000 reads sequenced. Asterisks indicate that the total number of unique reads sequenced was below 1,000,000 for the specified sample, therefore complexity estimates could not be generated. (C) The complexity of each sample, as measured by informative sequence content (D) The rate of contamination is compared by considering the rate of matching to mitochondrial consensus sequence. Error bars indicate the 95% confidence interval. Only samples that passed quality screening are shown. Plots showing comparisons with samples generated using Method P are shown in Supplementary Figure 2.

208   We therefore consider the overall complexity—the number of unique molecules contained

209   within a single library—using two metrics. In the first metric, we consider the proportion

210   of unique molecules sequenced in each sample, after down-sampling to 1,000,000

211   sequences that align to the 1.24 million SNPs targeted during capture (Figure 2B). This

212   is a useful metric for comparison between samples, as it is not biased by differences in

213   sequencing depth across samples. However, as this metric is calculated using sequence

214   data for samples that underwent targeted enrichment capture, a process that may

215   introduce bias into the data, we therefore also consider a second complexity metric, the

216   informative sequence content (Glocke and Meyer 2017). This metric quantifies the

217   relative proportion of molecules that were successfully amplified from each sample using

218   quantitative PCR (qPCR) analysis. The results are calibrated using the proportion of

219   endogenous molecules and average length of molecules measured in the shotgun

220   sequencing data, reflecting the number of sequences in the DNA extracts that can be

221   aligned to the human genome.

222

223   Neither complexity metric finds a statistically significant difference between complexity

224   measured in samples prepared using Method MDE versus Method WTR (p-value=0.792

225   and 0.107, for the first and second complexity metrics, respectively), suggesting that

226   using a minimally destructive extraction method does not result in loss of genetic data

227   when sampling from teeth (Figure 2B, Table 1). While we find no statistically significant

228   difference between samples prepared using Method MDE versus Method P using the first

229   complexity metric (p-value=0.091), we do detect a significant difference using the second

230   metric (p-value=0.043) (Supplementary Figure 2B-C). We note that the power of this

231   analyses is limited due to the low number of comparisons we were able to make (N=7),

232   therefore this comparison may warrant further study, particularly because previous

233   studies have found that the rates of ancient DNA preservation in cementum versus

234   petrous samples is dependent upon sample preservation (Hansen et al. 2017).

235   Contamination Rate

236   We were concerned that extracting ancient DNA directly from the outer layer of the tooth

237   root might result in a higher rate of contamination in the sample, especially due to the

238   increased potential for exposure of this region to contaminants during handling. Standard

239   sampling protocols typically involve the physical removal of the outermost layer of bone

240   or tooth prior to sampling, using a sanding disc or a sandblaster, while, in contrast, the

241   minimally destructive extraction method specifically targets this outer layer following a

242   superficial chemical (bleach) and brief (5-10 minute) ultraviolet decontamination. We

243   therefore compare the relative contamination rates between sampling methods using a

244   variety of metrics. First, we compare the rate of matching to the mitochondrial consensus

245   sequence (Fu et al. 2013). A minimum threshold of 95% is typically applied during

246   screening of ancient DNA for population genetic studies. We observe substantial

247   variability in contamination rate between and within individuals for all treatment methods

248   (Figure 2D, Table 1). While we detect a significant difference between mitochondrial

249   match to consensus rates between the MDE and WTR methods (p-value=0.004), the

250   average difference between these two methods is small (97.0% and 98.2%, respectively).

251   Further, we observe no significant difference between the Methods MDE and P (p=0.310)

252   (Supplementary Figure 2D).

253

254   Next, we estimate the autosomal rate of contamination, using the tool ContamLD

255   (Nakatsuka et al. 2020), which measures the breakdown of linkage disequilibrium in a

256   sequenced individual, a process which is accelerated by increased contamination. We

257   again estimate relatively low rates of contamination across all samples, and find no

258   significant difference in contamination rates between Methods MDE and WTR (p-

259   value=0.490) or between Methods MDE and P (p-value=0.893).

260

261   We also estimate contamination rates in the individuals who are identified as genetically

262   male using ANGSD (Korneliussen et al. 2014). We obtain low estimates of contamination

263   (≤2.5%) across all male samples (Table 1). Comparing the X-chromosome contamination

264   estimates for the 6 genetically male individuals for whom there was enough data to

265   produce estimates for both treatment types, we do not detect a significant difference

266   between the MDE and WTR Methods (p-value=0.293). Taken together these three

267   estimates of contamination suggest that, in practice, the UV and bleach decontamination

268   protocol used for the MDE Method performs similarly to the physical surface removal

269   decontamination steps implemented in the destructive protocols, and is sufficient to

270   produce ancient DNA data of analyzable quality.

271

272   We considered the read length distribution and frequency of C-to-T damage in the

273   terminal bases of reads that aligned to the human genome (hg19) that were obtained via

274   shotgun sequencing (i.e. pre-capture). Authentic ancient DNA is thought to consist of

275   characteristically short fragments, with very few reads longer than 100 base pairs (Sawyer

276   et al. 2012; Dabney et al. 2013b; Glocke and Meyer 2017), therefore the read length

277   distribution is used as a general metric to assess ancient DNA authenticity. We find that

278   all samples appear to have read length profiles characteristic for authentic ancient DNA

279   (Supplementary Figure 3) and we do not observe a significant difference in median length

280   of reads obtained using Method MDE and Method WTR (p-value=0.375). A weakly

281   significant difference is observed between reads obtained using Method MDE and P (p-

282   value=0.034) (Table 1), suggesting that there may be systematic differences between

283   DNA preservation in petrous and tooth samples.

284

285   Endogenous ancient DNA samples are also thought to exhibit a high rate of C-to-T

286   damage, particularly in the terminal bases. Using a partial or USER UDG treatment for

287   double stranded and single stranded libraries, respectively (Rohland et al. 2015;

288   Gansauge et al. in Prep), we removed this damage in the interior of each molecule, while

289   retaining it in the terminal bases. Therefore, we are able to use the frequency of these

290   errors to assess ancient DNA authenticity. For samples processed using Method MDE

291   and WTR (p-value=0.249) we observe no significant difference in the frequencies of C-

292   to-T damage in terminal bases at the 5' end of molecules that aligned to the human

293   genome (hg19), obtained via shotgun sequencing. However, the distribution of damage

294   rates in samples processed using Method P are significantly different to Method MDE (p-

295   value=0.028), with higher rates of damage observed in libraries produced using Method

296   P in most (8/9) cases, again suggesting that there may be systematic differences between

297   DNA preservation in petrous and tooth samples (Table 1, Supplementary Figure 4 & 5).

298

299   Finally, we were concerned that the use of parafilm to cover portions of the tooth roots

300   that we did not want expose to the extraction buffer could serve as a possible source of

301   contamination. We therefore created a parafilm extraction control, in which a small strip

302   of parafilm (comparable in size to that used for covering the tooth roots), was added to a

303   tube of extraction buffer and underwent sample processing along with the MDE samples

304   and regular extraction blanks. We observe very few reads associated with this parafilm

305   blank (Supplementary Table 1), suggesting that the use of parafilm does not serve as a

306   significant source of contamination in the MDE Method.

307   ## DISCUSSION

308   This minimally destructive sampling protocol enables extraction of ancient DNA from the

309   cementum portion of tooth roots that is of similar quality to ancient DNA obtained from

310   teeth using traditional, destructive sampling methods that rely on powder produced

311   through drilling or cutting and powdering. This is true with regards to both the amount of

312   DNA that it is possible to obtain and the levels of contamination detected in the samples.

313   In contrast, our results suggest that DNA sampled from the petrous bone exhibits more

314   complexity than DNA sampled from the tooth cementum, indicating that there is still

315   justification for choosing to sample from petrous bones over teeth when trying to

316   maximize the chances of successfully sequencing ancient DNA, particularly in cases

317   where sample preservation is poor—a circumstance in which ancient DNA sampled from

318   petrous has previously been found to be of higher quality than in cementum (Hansen et

319   al. 2017). However, the physical damage to the sampled tooth is substantially reduced

320   and the morphological integrity of the sampled tooth is retained when using this minimally

321   destructive sampling protocol, making this an optimal sampling method of teeth in cases

322   where sample preservation is of the highest priority.

323

324   One of the major concerns surrounding an extraction protocol that targets the outer

325   surface of an ancient sample is the potential for an increase in contamination, as this

326   outer surface may come in direct contact with various contaminants, particularly during

327   handling. Since the majority of samples selected for ancient DNA analysis have been

328   excavated and manipulated without any consideration for potential future genetic studies,

329   this is of particular concern. While destructive methods physically remove the outermost

330   layer of bones and teeth to reduce contamination, we instead applied a bleach and UV

331   decontamination procedure to the tooth before processing. We detected little difference

332   in contamination rates between samples processed using this minimally destructive

333   decontamination and sampling method and those processed using standard destructive

334   methods. Further, these results suggest that decontamination procedures that involve

335   wiping a sample with bleach do not significantly reduce DNA yields, as opposed to

336   previously proposed decontamination methods involving the soaking of the sample for an

337   extended period of time (e.g. Higgins et al. 2013). By targeting the outer cementum tooth

338   surface directly, this method maximizes the proportion of cementum matrix which is being

339   digested and minimizes the amount of dentine sampled when compared to other

340   cementum-targeting methods (Damgaard et al. 2015), which sample a significant

341   proportion of the inner dentine layer in addition to the cementum. Furthermore, we find

342   that parafilm can be used to protect portions of the tooth that users do not wish to sample

343   (i.e. the tooth crown) from exposure to extraction buffer, without increasing contamination

344   rates.

345

346   While these results show that this minimally destructive approach is a promising

347   alternative to destructive sampling methods that are traditionally applied to ancient teeth,

348   we stress that further research is needed to determine whether it is recommended to opt

349   for this sampling method in all circumstances. Particularly, we note that the majority of

350   teeth chosen for this analysis were of moderate to excellent preservation status. The two

351   most poorly preserved individuals included in this study contained too little DNA to allow

352   for comparisons to be made between Methods MDE and WTR, and the tooth roots

353   processed via Method MDE sustained damage during processing. Further study of the

354   utility of this method on less well-preserved teeth is therefore of great interest.

355

356   As the impact on dental morphology is minimal, this approach enables the preservation

357   of samples for future analyses. Previous studies have shown that exposure to the

358   chemicals used for ancient DNA extraction (mainly EDTA and proteinase K) do not affect

359   a specimen's suitability for subsequent biochemical analyses, such as radiocarbon (AMS

360   C14) dating (Korlević et al. 2018). Therefore, teeth processed using this minimally

361   destructive protocol would remain suitable for future biochemical analyses.

362

363   This minimally destructive extraction method drastically reduces the amount of physical

364   destruction caused by ancient DNA extraction, creating no holes or cuts in the sampled

365   tooth or bone, while also shortening the overall length of the extraction protocol, without

366   meaningfully increasing the amount of contamination. This method makes it possible to

367   extract ancient DNA from individuals that would otherwise be unavailable for ancient DNA

368   study due to the destructive nature of traditional sampling methods.

369   # METHODS

370   All ancient DNA analyses were performed in dedicated clean rooms at the University of

371   Vienna and Harvard Medical School. For individuals 1-10, skeletal sampling, preparation

372 and DNA extraction were performed at the University of Vienna. Library preparation,

373 targeted enrichment capture, and sequencing was performed at Harvard Medical School.

374 For individuals 11-30, skeletal sampling was performed at the University of Vienna, while

375 all other processing was performed at Harvard Medical School.

Sampling

377 We selected skeletal elements from 30 ancient individuals of varying age, geographic

378 origin, and degree of preservation for analysis (Table 1). From each individual, we

379 selected a single multi-rooted tooth for sampling. For the first 10 individuals, we also

380 selected a temporal bone for sampling. We UV irradiated each tooth in a cross-linker for

381 5 to 10 minutes on each side, in order to remove as much surface contamination as

382 possible. We then cut off the roots of each tooth using a diamond cutting disc and a hand-

383 held Dremel drill, treating each root separately in all subsequent analyses. From each

384 individual, we randomly selected one tooth root ("Method MDE") for minimally destructive

385 extraction. These tooth roots were subject to additional surface cleaning by wiping the

386 teeth clean with a 2% bleach solution and rinsing with 95% ethanol, followed by UV-

387 irradiation for 5 to 10 minutes on each side. We prepared the second set of tooth roots

388 ("Method WTR") by removing the extreme outer surface of each tooth root using a sanding

389 disc and drill, and milling the root in a Retsch MM400 mixer mill for a total of 60 seconds

390 with a 10 seconds break after 30 seconds to produce a powder. Additionally, we obtained

391 approximately 50mg of bone powder from the petrous portion of each of the 10 selected

392 temporal bones, using standard methods ("Method P") (Pinhasi et al. 2019).

DNA Extraction

We prepared selected tooth roots (Method MDE) for minimally destructive extraction by recording the initial weight of the tooth root, then isolating the targeted portion of the tooth root using parafilm (Supplementary Figure 6; see Supplementary Information 1 for a step-by-step description of the minimally destructive extraction method). We targeted the lower portion of the tooth root, where cellular cementum is concentrated. All other surfaces were wrapped in UV-decontaminated parafilm in order to prevent significant contact with the extraction buffer. The tooth roots were placed in 750 µL - 1 mL of extraction buffer (0.45 M EDTA, 0.25 mg/mL Proteinase K, pH 8.0; defined in Rohland and Hofreiter (2007) with the exposed portion pointing down, and incubated for 2.5 hours at 37°C, shaking gently. Following incubation, the roots were removed from the extraction buffer, which was then processed according to standard ancient DNA extraction procedures. Samples from individuals 1-10 underwent manual ancient DNA extraction, as described in Dabney et al. (2013a), with modifications. The MinElute columns were replaced with a preassembled spin column device (Roche, as described in Korlević et al. (2015)). We washed lysates with 650 µL of PE buffer (Qiagen) and spun at 6000 rpm for 1 minute. Following dry spin, we isolated the DNA by placing the spin column in a fresh 1.5 mL collection tube, and 25 µL TET buffer was pipetted onto the column's silica membrane, which was incubated at room temperature for 10 minutes, and then spun at maximum speed for 30 seconds. We repeated this step, producing a total of 50 uL of DNA extract. Samples from individuals 11-30 underwent robotic extraction following incubation, using the robotic protocol described in Rohland et al. (2018), using buffer D.

416   For samples processed using Methods WTR and P, sampled bone powders were

417   incubated overnight (~18 hours) in extraction buffer at 37°C, with gentle shaking. For

418   samples from individuals 1-10, up to 50mg bone powder was incubated in 1mL extraction

419   buffer, which then underwent manual extraction, as described above. For samples from

420   individuals 11-30, ~37 mg of bone powder was incubated in 750 μL extraction buffer, and

421   then underwent robotic extraction, as described above.

422

423   Negative controls were prepared alongside ancient DNA extracts for all extraction

424   batches. In each case, extraction buffer was added to an empty tube prior to incubation,

425   and the negative control was treated identically to all other samples during subsequent

426   processing. Additionally, we generated one parafilm extraction control, by incubating a

427   piece of UV-decontaminated parafilm in extraction buffer overnight in order to determine

428   whether the parafilm coverings used to protect the ends of the tooth roots might be a

429   potential source of contamination.

430

431   Following incubation in the extraction buffer, the roots were rinsed with 95% ethanol in

432   order to remove any remaining extraction buffer and air dried at room temperature for 24

433   hours. The samples were then re-weighed to assess the total amount of dental material

434   digested.

435   Library Preparation, Enrichment, and Sequencing

436   We prepared double-stranded (samples 1-10) or single-stranded (samples 11-30)

437   libraries from 10 µL of each extract using UDG-treatment methods, as described in

438   Rohland et al. (2015) and Gansauge et al. (in Prep), respectively. These methods remove

439 ancient DNA damage at the interior of each DNA sequence, while preserving

440 characteristic ancient DNA damage at the terminal ends of the molecules, to be used for

441 ancient DNA authentication during bioinformatic processing. We enriched libraries for

442 human DNA via targeted enrichment at 1.24 million SNP sites that are informative for

443 population genetic analyses (Fu et al. 2015; Haak et al. 2015; Mathieson et al. 2015).

444 Following enrichment, libraries were sequenced on an Illumina NextSeq500 machine,

445 with 2x76 or 2x101 cycles, with an additional 2x7 or 2x8 cycles used for identification of

446 indices, for double-stranded and single-stranded libraries, respectively.

Bioinformatic Processing

448 We trimmed molecular adapters and barcodes from sequenced reads, and the merged

449 paired end reads, requiring an overlap of 15 base pairs (allowing up to three mismatches

450 of low base quality (<20) or one mismatch of high base quality (≥20)) using custom

451 software (https://github.com/DReichLab/ADNA-Tools). We then aligned the merged

452 sequences to both the mitochondrial RSRS genome (Behar et al. 2012) and the hg19

453 human reference sequence using *samse* in bwa (v0.6.1) (Li and Durbin 2009). We

454 identified duplicate reads, defined as having the same start and end position and

455 orientation, and a shared DNA barcode (unique quadruple barcode combinations are

456 inserted during library preparation), and retained only the copy with the highest quality

457 sequence.

458

459 We assessed ancient DNA authenticity using several metrics. We used the tool

460 ContamMix (Fu et al. 2014) to determine the rate of matching between mitochondrial

461 reads and the consensus sequence. The tool ContamLD was used to estimate the rate

462 of contamination in the autosomes, based on the degree of breakdown of linkage

463 disequilibrium observed in each library relative to a panel of representative individuals

464 from the 1000 Genomes project (Nakatsuka et al. 2020). We determined the amount of

465 contamination in the X-chromosome for male individuals using the tool ANGSD

466 (Korneliussen et al. 2014). Finally, we estimated the rate of C-to-T substitution at the

467 terminal ends of molecules for each sample (Jónsson et al. 2013) and the lengths of

468 sequenced molecules were considered as metrics of DNA authenticity for each sample.

469

470 We assessed the quality of ancient DNA observed by measuring the percent of

471 endogenous (unique reads that align to the human genome), coverage (average number

472 of reads aligning to each of the 1.24 million targeted SNP sites), and overall complexity

473 of the sample—assessed by determining the proportion of unique reads sequenced, after

474 randomly down-sampling to 1,000,000 on-target reads, or by measuring the informative

475 sequence content (Glocke and Meyer 2017), in order to minimize bias caused by

476 differences in sequencing depth.

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DISCLOSURE DECLARATION

The authors declare no conflicts of interests.

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