

A Minimally Destructive Protocol for 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.

21 INTRODUCTION

22 Over the past decade, the field of ancient DNA has experienced a rapid increase in the
23 number of ancient genomes published each year (Slatkin and Racimo 2016) as a
24 consequence of advances in ancient DNA sampling (Gamba et al. 2014; Damgaard et al.
25 2015), extraction (Dabney et al. 2013a; Rohland et al. 2018), and enrichment (Carpenter
26 et al. 2013; Fu et al. 2013) techniques. As our ability to sequence large numbers of ancient
27 individuals has increased, discussions about the destructive nature of ancient DNA
28 sampling—which typically requires drilling or cutting and powdering ancient bones and
29 teeth—have become more prominent (Makarewicz et al. 2017; Prendergast and Sawchuk
30 2018; Sirak and Sedig 2019). The identification of the osseous inner ear, and specifically
31 the cochlea (located in the petrous portion of the temporal bone), as an optimal source of
32 ancient DNA (Gamba et al. 2014; Pinhasi et al. 2015; Pinhasi et al. 2019) is one of the
33 driving factors in this revolution, making it possible to access ancient DNA from
34 geographic regions with climatic conditions unfavorable to ancient DNA preservation.
35 However, accessing this optimal source of ancient DNA results in the destruction of the
36 inner ear morphology, which is a valuable source of morphological information (de León
37 et al. 2018). While there are protocols that reduce the destructive nature of sampling, by
38 sampling from the ossicles of the inner ear (Sirak et al. 2020) or performing targeted
39 drilling of the cochlea through the cranial base of complete or reconstructed crania (Sirak
40 et al. 2017), some destruction (including that of morphologically-informative inner ear
41 components) is inevitable. As a consequence, this and other less-invasive methods may
42 be considered unsuitable in cases where samples are of particular anthropological value
43 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 cemento-enamel 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).

100 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 | Glăvănești, 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 | Glăvănești, 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 | Ciurmai, 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 | .. |

| | | | | | | | | | | | | |
|----|-----|--|--------|--------|------|----|-------|--------|----------|-----------------|------------------|--------|
| | 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-Kápolnadbomb, 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.

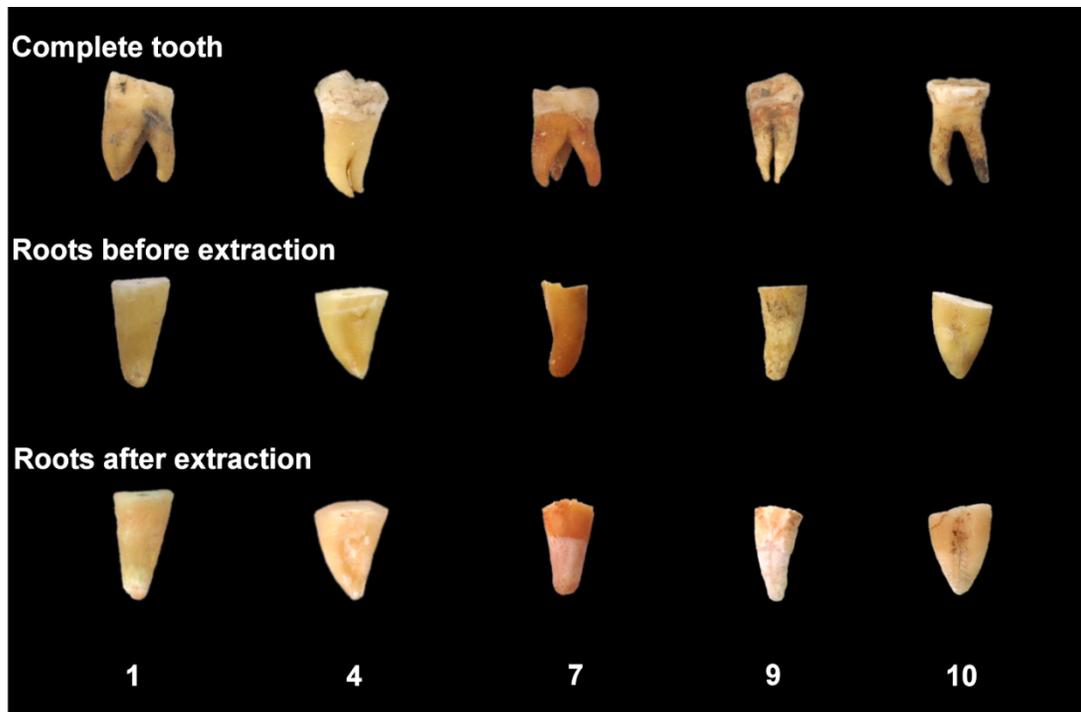
143 *Sampling/Extraction Methods: P- Powdered Petrous Bone (Pinhasi et al. 2019), standard extraction
144 (Dabney et al. 2013a); MDE- Tooth Root processed via Minimally Destructive Extraction; WTR- Whole
145 Tooth Root, powdered with standard extraction (Dabney et al. 2013a). Extracts for individuals 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.



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173
174

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.

175 Comparison of Minimally Destructive Extraction Protocol versus Powder- 176 Based Extraction Protocols

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

186 to denote significance which can only be achieved if there are a minimum of 6
187 comparisons per test.

188 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.

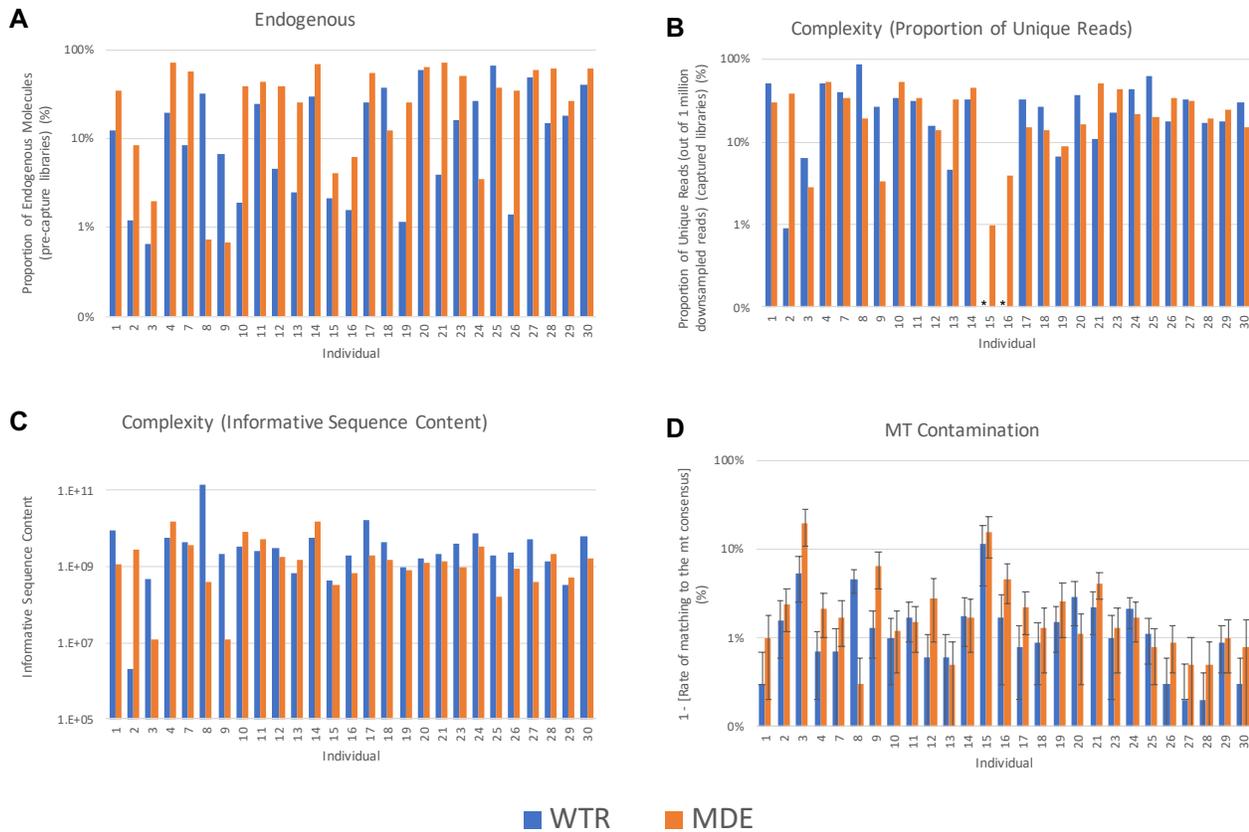


Figure 2: Sample Quality A comparison of the quality of data produced by WTR (Whole Tooth Root) and MDE (Minimally Destructive Extraction) Methods in samples that passed quality filtering. (A) The proportion of endogenous molecules in data obtained via shotgun sequencing. (B) The complexity of each sample, 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 ($\leq 2.5\%$) 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.

376 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).

393 DNA Extraction

394 We prepared selected tooth roots (Method MDE) for minimally destructive extraction by
395 recording the initial weight of the tooth root, then isolating the targeted portion of the tooth
396 root using parafilm (Supplementary Figure 6; see Supplementary Information 1 for a step-
397 by-step description of the minimally destructive extraction method). We targeted the lower
398 portion of the tooth root, where cellular cementum is concentrated. All other surfaces were
399 wrapped in UV-decontaminated parafilm in order to prevent significant contact with the
400 extraction buffer. The tooth roots were placed in 750 μ L - 1 mL of extraction buffer (0.45
401 M EDTA, 0.25 mg/mL Proteinase K, pH 8.0; defined in Rohland and Hofreiter (2007) with
402 the exposed portion pointing down, and incubated for 2.5 hours at 37°C, shaking gently.
403 Following incubation, the roots were removed from the extraction buffer, which was then
404 processed according to standard ancient DNA extraction procedures. Samples from
405 individuals 1-10 underwent manual ancient DNA extraction, as described in Dabney et al.
406 (2013a), with modifications. The MinElute columns were replaced with a preassembled
407 spin column device (Roche, as described in Korlević et al. (2015)). We washed lysates
408 with 650 μ L of PE buffer (Qiagen) and spun at 6000 rpm for 1 minute. Following dry spin,
409 we isolated the DNA by placing the spin column in a fresh 1.5 mL collection tube, and 25
410 μ L TET buffer was pipetted onto the column's silica membrane, which was incubated at
411 room temperature for 10 minutes, and then spun at maximum speed for 30 seconds. We
412 repeated this step, producing a total of 50 μ L of DNA extract. Samples from individuals
413 11-30 underwent robotic extraction following incubation, using the robotic protocol
414 described in Rohland et al. (2018), using buffer D.

415

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.

447 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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487 DISCLOSURE DECLARATION

488 The authors declare no conflicts of interests.

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