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Optimized bone sampling protocols for the retrieval of ancient DNA from archaeological remains

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TITLE:

Optimized Bone Sampling Protocols for the Retrieval of Ancient DNA from Archaeological Remains

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KEYWORDS:

archaeogenetics, aDNA, bone sampling, forensics, DNA sampling, bioanthropology

SUMMARY:

The protocol presents a series of best practice protocols for the collection of bone powder from eight recommended anatomical sampling locations (specific locations on a given skeletal element) across five different skeletal elements from medieval individuals (radiocarbon dated to a period of ca. 1040–1400 CE, calibrated 2-sigma range).

ABSTRACT:

The methods presented here seek to maximize the chances for the recovery of human DNA from ancient archaeological remains while limiting input sample material. This was done by targeting anatomical sampling locations previously determined to yield the highest amounts of ancient DNA (aDNA) in a comparative analysis of DNA recovery across the skeleton. Prior research has suggested that these protocols maximize the chances for the successful recovery of ancient human and pathogen DNA from archaeological remains. DNA yields were previously assessed by Parker et al. 2020 in a broad survey of aDNA preservation across multiple skeletal elements from 11 individuals recovered from the medieval (radiocarbon dated to a period of circa (ca.) 1040–1400 CE, calibrated 2-sigma range) graveyard at Krakauer Berg, an abandoned medieval settlement near Peißen Germany. These eight sampling spots, which span five skeletal elements (*pars petrosa*, permanent molars, thoracic vertebra, distal phalanx, and talus) successfully yielded high-quality ancient human DNA, where yields were significantly greater than the overall

average across all elements and individuals. Yields were adequate for use in most common downstream population genetic analyses. Our results support the preferential use of these anatomical sampling locations for most studies involving the analyses of ancient human DNA from archaeological remains. Implementation of these methods will help to minimize the destruction of precious archaeological specimens.

INTRODUCTION:

The sampling of ancient human remains for the purposes of DNA recovery and analysis is inherently destructive[1–4]. The samples themselves are precious specimens and morphological preservation should be preserved wherever possible. As such, it is imperative that sampling practices be optimized to both avoid unnecessary destruction of irreplaceable material and to maximize the probability of success. Current best practice techniques are based on a small cohort of studies limited to either forensic surveys[5,6], studies of ancient specimens where the development of optimal sampling is not the direct aim of the study[7], or dedicated studies utilizing either non-human remains[8] or targeting a very small selection of anatomical sampling locations (used here to denote a specific area of a skeletal element from which bone powder, for use in downstream DNA analyses, was generated)[9,10]. The sampling protocols presented here were optimized in the first large-scale systematic study of DNA preservation across multiple skeletal elements from multiple individuals[11]. All samples stemmed from skeletal elements recovered from 11 individuals excavated from the church graveyard of the abandoned medieval settlement of Krakauer Berg near Peißen, Saxony-Anhalt, Germany (see **Table 1** for detailed sample demographics) and, as such, may need modification for use with samples outside of this geographical/temporal range.

[Insert **Table 1** here]

These protocols allow for a relatively straightforward and efficient generation of bone powder from eight anatomical sampling locations across five skeletal elements (including the *pars petrosa*) with limited laboratory-induced DNA contamination. Of these five skeletal elements, seven anatomical sampling locations found on four skeletal elements have been determined to be viable alternatives to the destructive sampling of the petrous pyramid[11,12]. These include the cementum, dentin, and pulp chamber of permanent molars; cortical bone gathered from the superior vertebral notch as well as from the body of thoracic vertebrae; cortical bone stemming from the inferior surface of the apical tuft and shaft of the distal phalanges; and the dense cortical bone along the exterior portion of the tali. While there are several widely applied methods for the sampling of the *pars petrosa*[4,12–14], dentin, and the dental pulp chamber[1,2,15], published methods describing the successful generation of bone powder from the cementum[16], vertebral body, inferior vertebral notch, and talus can be difficult to obtain. As such, here we demonstrate optimized sampling protocols for the petrous pyramid (step 3.1); cementum (step 3.2.1), dentin (step 3.2.2), and dental pulp (step 3.2.3) of adult molars; cortical bone of the vertebral body (step 3.3.1) and superior vertebral arch (step 3.3.2); the distal phalanx (step 3.4); and the talus (step 3.5) in order to make the effective use of these skeletal elements for both aDNA and forensic research more widely accessible.

PROTOCOL:

All research presented herein was performed in compliance with the guidelines set forth by the Max Planck Institute for the Science of Human History, Jena, Germany for working with ancient human remains. Before performing any steps of this protocol ensure to adhere to all local/state/federal ethical requirements pertaining to both obtaining permission for the scientific study and use of human remains for destructive sampling in your area. All procedures/chemical storage should be performed according to individual institutional safety guidelines.

1. Considerations before sample processing

1.1. Treat samples with care as ancient remains are an irreplicable and finite resource (e.g., sampling should be as minimally wasteful as possible, and all remains returned to their respective and lawful providers if possible).

1.2. Perform all steps in a clean-room environment, preferably at a dedicated ancient DNA facility[17–19]. Use personal protective equipment (PPE) consisting of sterile microporous coveralls with hood, sterile gloves (two pairs), surgical mask, protective eyewear, and sterile boots or non-slip shoes with sterile covers (see **Table of Materials**). Change gloves frequently, especially between samples.

1.3. Clean and disinfect all equipment and surfaces thoroughly with bleach/DNA decontamination solution/ethanol and UV irradiation (wavelength: 254 nm) where possible (e.g., drill bits, drills, vices/clamps, etc.). Finally, it is highly recommended to take regular ergonomic breaks (every 2–3 h if possible) to avoid over-exhaustion due to the clean-room environment.

NOTE: All skeletal remains should be appropriately documented (e.g., photographed, weighed, and if possible micro-CT scanned, 3D imaged, etc.) before sampling (protocols for appropriate documentation are not covered in this manuscript). All sampling protocols may be paused between sampling iterations and the samples can be stored indefinitely in a dry, temperature controlled (25 °C), sterile environment.

2. Pretreatment

2.1. Decontaminate all anatomical sampling locations prior to bone powder generation to minimize the risk of contamination[18].

NOTE: The efficacy of bleach and/or surface removal (see NOTE in step 3.3.2 for surface removal steps) for sample decontamination is still a matter of debate among aDNA researchers[8,19–25] as both may influence overall DNA yields, especially in highly degraded samples. As such, the following steps are considered optional and are included here as they were used in all samples to generate the representative results presented in this paper. It is recommended that the use of these pre-treatment protocols be determined on a case-by-case basis based on the molecular application, age, rarity, and level of morphological degradation of each sample set.

2.1.1. Perform all sampling in a dedicated clean room under a UV light equipped polymerase chain reaction (PCR) hood or biosafety cabinet with airflow turned off. Spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments.

2.1.2. Ensure all bone fragments are recovered (for repatriation) before disposing of the foil. Change the foil between the treatment of each skeletal element. Dispose of used foil in an autoclavable biohazard bag/receptacle.

2.1.3. Remove as much loose dirt/detritus as possible from anatomical sampling locations by gently wiping the area with a lint-free dry sterile wipe (see **Table of Materials**). Dispose of the wipes in autoclavable biohazard bags or receptacles.

2.1.4. Decontaminate the cleaned surface by wiping with a sterile wipe moistened with diluted commercial bleach (~0.01% v/v, diluted with ultrapure DNase/RNase free water) and allow to incubate for 5 min. Dispose of the wipes in autoclavable biohazard bags or receptacles.

CAUTION: Bleach is a highly corrosive and reactive chemical; hence appropriate safety precautions should be in place before its use.

2.1.5. Remove as much residual bleach as possible from the anatomical sampling location with a sterile wipe moistened with ultrapure DNase/RNase-free water. Dispose of the wipes in autoclavable biohazard bags or receptacles.

2.1.6. Expose all cleaned anatomical sampling locations to UV radiation for 30 min (wavelength: 254 nm), and then allow to dry fully at room temperature. Ensure that the anatomical sampling locations are completely dry before proceeding with sampling or returning to storage to not only make bone powder generation easier but also to prevent further degradation of the sample (e.g., mold).

CAUTION: Exposure to UV radiation can be harmful to the eyes.

2.1.7. Move immediately to sampling or store skeletal elements in a dry, temperature controlled (25 °C) sterile environment.

3. **Bone powder generation**

NOTE: The following protocols are intended for use in DNA extraction following the Dabney et al. 2019 protocol[26].

3.1. Sampling of *pars petrosa*

NOTE: This protocol is adapted from procedures described in Pinhasi et al. 2019[4] and is presented here for ease of use. This protocol does not represent the current, least destructive method for the sampling of *pars petrosa*. As such, it is recommended to use the protocol described by Sirak

et al. 2017[13] or Orfanou et al. 2020[14] for samples where morphological preservation is of maximum importance.

3.1.1. Perform all sampling in a dedicated clean room under a UV light equipped PCR hood or biosafety cabinet (wavelength: 254 nm) with airflow turned off. Spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments.

3.1.2. Ensure all bone fragments and as much powder as possible is recovered (for repatriation) before disposing of foil. Change the foil between each sampling. Dispose of the used foil in an autoclavable biohazard bag/receptacle.

3.1.3. Secure the dry, decontaminated element using a sterilized clamp or vice.

3.1.4. Cut the *pars petrosa* in half along the superior *sulcus petrosus* (see **Figure 1**) using a standard jeweler's saw equipped with a 0.6 mm blade (see **Table of Materials**) at medium speed to avoid overheating (see NOTE below step 3.1.6).

CAUTION: The *pars petrosa* is very dense, and as such may be difficult to cut. Take care to keep the element securely clamped to avoid injury. Dispose of any broken saw blades in the appropriate sharps' receptacle.

3.1.5. Remove the petrous portions from the clamp and any loose material.

3.1.6. Place weigh paper in a sterile weighing boat

3.1.7. Hold the petrous portion over the weigh paper, cut side tilted toward the weighing tray. Drill into the dense cortical bone between the facial canal and mastoid antrum (appears shinier than the surrounding material, see **Figure 1**) using dental drill equipped with a small gauge bit (see **Table of Materials**) and set to medium speed, medium torque to produce bone powder.

NOTE: Drilling/Cutting should be done in short bursts at low to medium speeds to avoid overheating the bone and potentially destroying/damaging DNA. Anecdotally, when the dense portion of the petrous begins to overheat a smell described as cooking bacon may be observed. Cease drilling/sawing immediately and allow the bone to rest until sufficiently cool before resuming.

3.1.8. Repeat drilling until approximately 50–100 mg of powder is collected in the weigh paper, as measured using an enclosed balance accurate to at least 0.01 mg (see **Table of Materials**).

NOTE: Where possible it is suggested to gather 100 mg of bone powder to allow for two replicate DNA extraction of 50 mg each. However, this may not always be possible based on either limitation of the anatomical sampling locations themselves (e.g., the distal phalanx, dental pulp chamber) or the need for morphological preservation. For other locations, such as the cementum, considerably less than 50 mg of the material may be available. However, the

cementum, dental pulp chamber, and distal phalanx have all been shown to yield significant endogenous DNA[11,27,28], despite lower initial input of bone powder from the extraction process.

3.1.9. Transfer powder from the weigh paper to a 2 mL labeled low-bind, safe-lock tube for extraction or storage. Store samples at -20 °C, indefinitely.

3.1.10. Store remaining bone/excess powder in a dry, temperature controlled (25 °C) sterile environment until return/repatriation can be completed.

3.1.11. Dispose of all waste in autoclavable biohazard bags or receptacles. Sterilize/decontaminate all reusable equipment (e.g., clamps, drill bits, drills, saws, etc.) using bleach/DNA decontamination solution/ethanol and UV (wavelength: 254 nm) exposure, as applicable, between each sampling.

[Insert **Figure 1** here]

3.2. Sampling of permanent molars

NOTE: For the sampling of permanent molars, pre-select *in situ* molars with fused roots and ideally void of caries, cracks in the enamel, or excessive wear for best results. Remove any dental calculus sampling and store at -20 °C for possible future analyses of the oral microbiome (procedure not covered here).

3.2.1. Sampling of the cementum

3.2.1.1. Perform all sampling in a dedicated clean room under a UV light equipped PCR hood or biosafety cabinet (wavelength: 254 nm) with airflow turned off. Spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments.

3.2.1.2. Ensure all bone fragments and as much powder as possible are recovered (for repatriation) before disposing of foil. Change the foil between each sampling. Dispose of used foil in an autoclavable biohazard bag/receptacle.

3.2.1.3. Place a sheet of weigh paper into a sterile weighing tray.

3.2.1.4. Hold/secure the decontaminated molar by the enamel, root down, over a weighing tray using a hand-held clamp such as an adjustable wrench (see **Table of Materials**).

3.2.1.5. Equip a dental drill with a diamond-edged circular cutting wheel. With the drill set to a medium speed/torque setting, lightly touch the edge of the bit to the root at an angle of approximately -20°.

3.2.1.6. Scrape downward into the tray to remove/collect the yellow, outermost material from the root (cementum). Stop collection when the lighter (white) material of the dentin becomes visible.

NOTE: It is important to match the direction of rotation of the cutting bit in relation to the collection tray to avoid the powder becoming aerosolized and potentially wasting the sample by missing the tray entirely. The cementum is particularly rich in DNA; however, typical yields of material are much smaller than other anatomical sampling locations (~7–20 mg)[11,27,28].

3.2.1.7. Record mass of powder collected in weigh paper using an enclosed balance accurate to at least 0.01 mg (see **Table of Materials**).

3.2.1.8. Transfer powder from the weigh paper to a 2 mL low-bind, safe lock tube for extraction. Store at -20 °C, indefinitely.

3.2.2. Sampling of the pulp chamber

3.2.2.1. After the cementum has been collected (if desired), section the molar along the cemento-enamel junction using a jeweler's saw to remove the crown (see **Figure 2**).

3.2.2.2. Place a new sheet of weigh paper in a new weighing tray.

3.2.2.3. Secure the crown section in a handheld clamp or vice, over the weighing tray. Hold cut side tilted downward and drill/scrape material as the first pass with a dental drill equipped with a small gauge drilling bit (see **Table of Materials**) along the edges of the pulp chamber within the crown portion (see **Figure 2**).

NOTE: Only the first pass of the interior of the pulp chamber is to be collected and labeled as pulp material (5–15 mg typical yield), anything deeper into the tooth is considered dentin.

3.2.2.4. Turn the tooth with the inferior portion facing down, tap the clamp with a hammer, and collect the liberated powder on the weigh paper.

3.2.2.5. Record the weight of the powder collected in the weigh paper using an enclosed balance accurate to at least 0.01 mg (see **Table of Materials**).

3.2.2.6. Transfer powder from the weigh paper to a 2 mL low-bind, safe-lock tube for extraction. Store at -20 °C, indefinitely.

3.2.3. Sampling of the dentin

3.2.3.1. Place a new sheet of weigh paper in a new weighing tray.

3.2.3.2. Hold the crown section over the weighing tray (as per step 3.2.2.3), drill out and collect further 50–100 mg of dentin as measured using an enclosed balance accurate to 0.01 mg (see **Table of Materials**) from the interior of the pulp chamber in the same manner for further dentin sampling (see **Figure 2**).

3.2.3.3. Transfer bone powder from the weigh paper to a 2 mL low-bind, safe-lock tube for extraction. Store at -20 °C, indefinitely.

3.2.3.4. Store the remaining tooth pieces/excess powder in a dry, temperature controlled (25 °C) sterile environment until return/repatriation can be completed.

3.2.3.5. Dispose of all waste in autoclavable biohazard bags or receptacles. Sterilize/decontaminate all reusable equipment (e.g., clamps, drill bits, drills, saws, etc.) using bleach/DNA decontamination solution/ethanol and UV (wavelength: 254 nm) exposures as applicable, between each sampling.

[Insert **Figure 2** here].

3.3. Sampling of the thoracic vertebrae

3.3.1. Sampling of the vertebral body

3.3.1.1. Perform all sampling in a dedicated clean room under a UV light equipped PCR hood or biosafety cabinet (wavelength: 254 nm) with airflow turned off. Spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments.

3.3.1.2. Ensure all bone fragments and as much powder as possible are recovered (for repatriation) before disposing of foil. Change the foil between each sampling. Dispose of used foil in an autoclavable biohazard bag/receptacle.

3.3.1.3. Place a small sheet of weigh paper into a standard weighing tray.

3.3.1.4. Secure the vertebrae with a clamp or hand vice, with the vertebral body outward.

3.3.1.5. Hold the vertebrae over the weighing tray with the vertebral body tilted downward. Using a dental drill equipped with a small gauge drilling bit (see **Table of Materials**) set to low-speed high torque, drill along the outermost rim (inferior and superior) of the cortical bone surrounding the cancellous inner tissue of the vertebral body (see **Figure 3**).

3.3.1.6. Scrape the bit against the cortical layer over a standard weighting tray until 50–100 mg of material is collected, as measured using an enclosed balance accurate to 0.01 mg (see **Table of Materials**).

3.3.1.7. Transfer bone powder from the weigh paper to a 2 mL low-bind, safe lock tube for extraction. Store at -20 °C, indefinitely.

3.3.2. Sampling of the superior vertebral arch

NOTE: This step is optional. Remove and discard the outermost layer of the cortical bone of the superior vertebral arch using a dental drill equipped with a small gauge drilling bit (see **Table of Materials**) by scraping it along the surface[19]. This is not suggested for sampling from the vertebral body, as the layer of cortical bone is generally very thin and likely to be entirely depleted by this process (see NOTE in section 2).

3.3.2.1. Place a small sheet of weigh paper into a standard weighing tray.

3.3.2.2. Secure the vertebrae in a hand clamp/vice with the vertebral process outward, superior aspect down.

3.3.2.3. While holding the vertebrae, superior aspect down, over a weighing tray, drill upwards into the center of the V shaped notch formed by the fusion of the spinous process with the lamellae (see **Figure 3**) using a dental drill with a small gauge bit (see **Table of Materials**) set to low speed and high torque.

3.3.2.4. Cease drilling when there is a noticeable drop in resistance. Change the drilling position slightly and repeat until 50–100 mg of bone powder is collected, as measured using an enclosed balance accurate to 0.01 mg (see **Table of Materials**).

3.3.2.5. Transfer bone powder from the weigh paper to a 2 mL low-bind tube for extraction. Store at -20 °C, indefinitely.

3.3.2.6. Store remaining bone/excess powder in a dry, temperature controlled (25 °C) sterile environment until return/repatriation.

3.3.2.7. Dispose of all waste in autoclavable biohazard bags or receptacles. Sterilize/decontaminate all reusable equipment (e.g., clamps, drill bits, drills, saws, etc.) using bleach/DNA decontamination solution/ethanol and UV (wavelength: 254 nm) exposure, as applicable, between each sampling.

[Insert **Figure 3** here].

3.4. Sampling of the distal phalanx

NOTE: This step is optional. Remove and discard the outermost layer of the cortical bone of the shaft and/or apical tuft using a dental drill equipped with a small gauge drilling bit by scraping it along the surface[19]. This may not be possible for samples with excessively thin cortical bone or juvenile remains (see NOTE in section 2).

3.4.1. Perform all sampling in a dedicated clean room, under a UV light equipped PCR hood or biosafety cabinet (UV wavelength: 254 nm) with airflow turned off. Spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments.

3.4.2. Ensure all bone fragments and as much powder as possible are recovered (for repatriation) before disposing of foil. Change the foil between each sampling. Dispose of used foil in an autoclavable biohazard bag/receptacle.

3.4.3. Place a small sheet of weigh paper into a standard weighing tray.

3.4.4. Secure the sample in handheld clamp/vice, superior side upwards.

3.4.5. Hold the sample over the weighing tray, collect bone powder from the cortical bone from the inferior side of the apical tuft and shaft by drilling through the outermost dense layers (see **Figure 4**) using a dental drill equipped with a small gauge drilling bit (see **Table of Materials**).

3.4.6. Cease drilling when there is a marked decrease in resistance, as this signifies lighter, cancellous material. Repeat this process, radiating outward from the initial drilling until at least 50–100 mg of bone powder is collected, as measured using an enclosed balance accurate to 0.01 mg (see **Table of Materials**).

3.4.7. Transfer bone powder from the weigh paper to a 2 mL low-bind, safe-lock tube for extraction. Store at -20 °C, indefinitely.

3.4.8. Store the remaining bone/excess powder in a dry, temperature controlled (25 °C) sterile environment until return/repatriation.

3.4.9. Dispose of all waste in autoclavable biohazard bags or receptacles. Sterilize/decontaminate all reusable equipment (e.g., clamps, drill bits, drills, saws, etc.) using bleach/DNA decontamination solution/ethanol and UV exposure, as applicable, between each sampling.

NOTE: For smaller samples (e.g., juvenile samples) there may be considerably less than the suggested 50–100 mg of cortical bone available to sample. However, even in low quantities, this anatomical sampling location has been shown to be particularly rich in DNA[11].

[Insert **Figure 4** here]

3.5. Sampling of the Talus

3.5.1. Perform all sampling in a dedicated clean room under a UV light equipped PCR hood or biosafety cabinet (wavelength: 254 nm) with airflow turned off. Spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments.

3.5.2. Ensure all bone fragments and as much powder as possible are recovered (for repatriation) before disposing of foil. Change the foil between each sampling. Dispose of used foil in an autoclavable biohazard bag/receptacle.

3.5.3. Place a small sheet of weigh paper into a standard weighing tray.

3.5.4. Secure the sample in handheld clamp/vice, dome upwards.

3.5.5. Hold the talus, dome upward, and medial surface toward the collector, over the weighing tray. Scrape cortical bone from the neck of the talus to a depth of ~1 mm (see **Figure 5**) using a dental drill with a low gauge bit (see **Table of Materials**) set to low speed and high torque.

3.5.6. Change the drilling position slightly and repeat until approximately 50–100 mg of bone powder is collected, as measured using an enclosed balance accurate to 0.01 mg (see **Table of Materials**).

3.5.7. Transfer bone powder from the weigh paper to a 2 mL low-bind tube for extraction. Store at -20 °C, indefinitely.

3.5.8. Store the remaining bone/excess powder in a dry, temperature controlled (25 °C) sterile environment until return/repatriation can be completed.

3.5.9. Dispose of all waste in autoclavable biohazard bags or receptacles. Sterilize/decontaminate all reusable equipment (e.g., clamps, drill bits, drills, saws, etc.) using bleach/DNA decontamination solution/ethanol and UV (wavelength: 254 nm) exposure, as applicable, between each sampling.

[Insert **Figure 5** here].

NOTE: The talus has very little cortical bone (a thin outer layer). The material should not only be collected from the surface but also the underlying dense layer of cancellous bone.

REPRESENTATIVE RESULTS:

In a separate study[11], DNA was extracted from bone powder generated from each anatomical sampling location in 11 individuals, using a standard DNA extraction protocol optimized for short fragments from calcified tissue[2]. Single-stranded libraries were then produced[28] and sequenced on a HiSeq 4000 (75 bp paired-end) to a depth of ~20,000,000 reads per sample. The resulting sequence data was then evaluated for endogenous human DNA content using the EAGER pipeline[29] (BWA settings: Seed length of 32, 0.1 mismatch penalty, mapping quality filter of 37). All representative results are reported using the same metrics as Parker et al. 2020[11] for consistency. Libraries from the powdered portions of the *pars petrosa* yielded, on average, higher endogenous DNA than any of the other 23 anatomical sampling locations surveyed (**Figure 6A–B)**. The seven additional anatomical sampling locations presented in this protocol (the

cementum, first pass of the dental pulp chamber, and dentin of permanent molars; cortical bone from the vertebral body and superior vertebral arch of the thoracic vertebra; cortical bone from the apical tuft of the distal phalanx; and cortical bone from the neck of the talus) produced the next highest yields (with no statistical significance between these anatomical sampling locations; **Figure 6A–B; Supplemental File 1:** EndogenousDNAPreCap). These alternative locations all consistently produced DNA yields adequate for standard population genetics analyses such as mitochondrial analyses and single nucleotide polymorphism (SNP) analyses. Duplication rates in libraries stemming from all anatomical sampling locations were low (cluster factors < 1.2 on average, calculated as the ratio of all mapping reads to unique mapping reads, **Table 2; Supplemental File 1**: ClusterFactor), indicating that all libraries screened were of very high complexity. Similarly, average exogenous human DNA contamination estimates were low, averaging < 2% (X chromosome contamination in males, n = 7, as reported by the ANGSD[30] pipeline) in all anatomical sampling locations except for the superior vertebral arch (average estimated contamination: 2.11%, with one sample removed as an outlier; KRA005: 19.52%, see **Table 2; Supplemental File 1**: Xcontamination). Average fragment length (after filtering to remove all reads < 30 bp) was lowest in the material collected from the dental pulp chamber and dentin, with no significant variation among other anatomical sampling locations (55.14 bp and 60.22 bp, respectively in comparison to an average median of 62.87, pair-wise p-values < 0.019, **Table 2; Supplemental File 1**: AvgFragLength). Additionally, the teeth and thoracic vertebrae each contain multiple anatomical sampling locations where high endogenous DNA recovery was observed, making them particularly suitable as alternatives to the *pars petrosa*.

[Insert **Figure 6** here]

[Insert **Table 2** here]

Code availability

All analyses programs and R modules used in the analyses of this manuscript are freely available from their respective authors. All custom R code is available by request.

Data availability

All raw data used in the calculation of representative results is freely available in the European Nucleotide Archive ENA data repository (accession number PRJ-EB36983) or supplemental materials of Parker, C. et al.[11].

FIGURE AND TABLE LEGENDS:

Figure 1: Temporal bone including the *pars petrosa*. (**A**) Sample pre-cutting showing the locations of the petrous pyramid and the *sulcus petrosa.* (**B**) Petrous portion post-cutting highlighting the dense areas to be drilled.

Figure 2: Permanent molar pre-sampling. (**A**) Pre-treated molar prior to sampling, showing crown, cementum (yellowish layer of the root), and the cutting site at the cemento-enamel junction. (**B**) The same molar post-cementum collection, showing the cut site at the cemento-

enamel junction. (**C**) Molar post-cutting and sampling showing anatomical sampling locations for the dental pulp chamber and dentin within the crown.

Figure 3: Vertebral body and superior vertebral arch cortical bone anatomical sampling locations of the thoracic vertebra.

Figure 4: Distal phalanx showing the locations of dense cortical bone along the shaft and inferior side of the apical tuft.

Figure 5: Sampling area of the talus for cortical bone recovery.

Figure 6: Human DNA content for all screened samples. Black lines represent the overall mean, while red lines represent the median (solid: human DNA proportion, dashed: mapped human reads per million reads generated). Individual anatomical sampling locations with an average human DNA proportion higher than the overall mean (8.16%) are colorized in all analyses. (**A**) The proportion of reads mapping to the hg19 reference genome. The blue dashed line represents the theoretical maximum given the pipeline's mapping parameters (generated using Gargammel[31] to simulate a random distribution of 5,000,000 reads from the hg19 reference genome with simulated damage). Individual means (black X) and medians (red circle) are reported for those samples with a higher average human DNA proportion than the overall mean. Confidence intervals indicate upper and lower bounds excluding statistical outliers. (**B**) The number of unique reads mapping to the hg19 reference genome per million reads of sequencing effort (75 bp paired end). Confidence intervals indicate upper and lower bounds excluding statistical outliers. This figure has been adapted from Parker, C. et al. 2020[11].

Table 1: Genetically determined sex, archaeologically determined estimated age at death, and radiocarbon dating (^{14}C Cal 2-sigma) for all the 11 individuals sampled. This table has been adapted from Parker, C. et al. 2020[11].

Table 2: Average duplication levels (mapping reads/unique reads), average and median fragment lengths, and X chromosome contamination estimates for all anatomical sampling locations. Error reported as the standard error of the mean. This table has been adapted from Parker, C. et al. 2020[11].

DISCUSSION:

Current practice in ancient human population genetics is to preferentially sample from the *pars petrosa* (step 2.1) whenever possible. However, the *pars petrosa* can be a difficult sample to obtain, as it is highly valued for a myriad of skeletal assessments (e.g., population history[32], the estimation of fetal age at death[33], and sex determination[34]), and, historically, sampling of the *pars petrosa* for DNA analysis can be highly destructive[3–4] (including the protocol presented here, although new, minimally invasive protocols[13,14] have now been widely adopted to alleviate this concern). This is compounded by the fact that, until very recently, a large-scale, systematic study of human DNA recovery across the skeleton had not been attempted[11], making finding an appropriate sampling strategy when the petrous pyramid is unavailable challenging.

The protocols presented here help to alleviate that challenge by providing a set of optimized procedures for DNA sampling from archaeological/forensic skeletal remains including the *pars petrosa* as well as seven alternate anatomical sampling locations across four additional skeletal elements. The critical steps included are all intended to minimize the possibility of DNA loss/damage due to either inefficient sampling (steps 2.1.6 and 3.2.1.3) or overheating of samples during drilling/cutting (step 3.1.6). Additionally, it has been noted throughout the protocol that it may be necessary to modify/omit the pre-treatment steps to ensure the best performance in highly degraded samples. It should also be noted that even among the selected elements presented here, there remain several possible alternative sampling techniques (particularly for the *pars petrosa*[13,14]), as well as ample room for further optimization of the underexploited anatomical sampling locations presented here *(*i.e., the talus: step 2.5 and the vertebrae: step 2.3).

It is also important to keep in mind that these protocols have been designed and tested using ancient juvenile-adult remains of high quality (good morphological preservation) for the purposes of endogenous human DNA analyses. The results presented may not extend to more highly degraded materials, other preservation contexts, infant remains, non-human remains, or studies of pathogens or the microbiome, as a greater exploration into the use of these protocols in additional contexts is still needed. Additionally, the alternative skeletal elements presented here (the teeth, vertebrae, distal phalanx, and tali) may be challenging to assign to a single individual among commingled remains, necessitating sampling from multiple elements to ensure a single origin. Despite these limitations, making these protocols widely available can help alleviate some of the heterogeneity surrounding sample selection and processing by providing a generalized and quantitatively optimized framework for use in a wide range of future aDNA/forensic studies on human remains.

ACKNOWLEDGMENTS:

The authors would like to thank the laboratory staff of the Max Planck Institute for the Science of Human History for their help in the development and implementation of these protocols. This work would not have been possible without the input and hard work of Dr. Guido Brandt, Dr. Elizabeth Nelson, Antje Wissegot, and Franziska Aron. This study was funded by the Max Planck Society, the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program under grant agreements No 771234 – PALEoRIDER (WH, ABR) and Starting Grant No. 805268 CoDisEASe (to KIB).

DISCLOSURES:

The authors have no conflicts of interest to report.

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Figure 1

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A.

B.

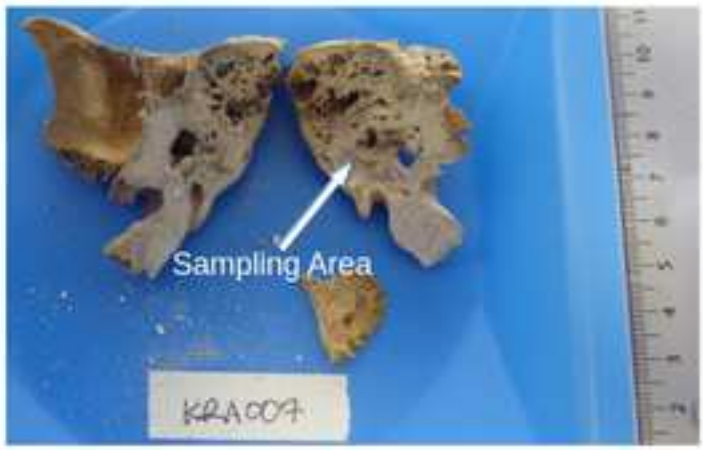

Figure 2

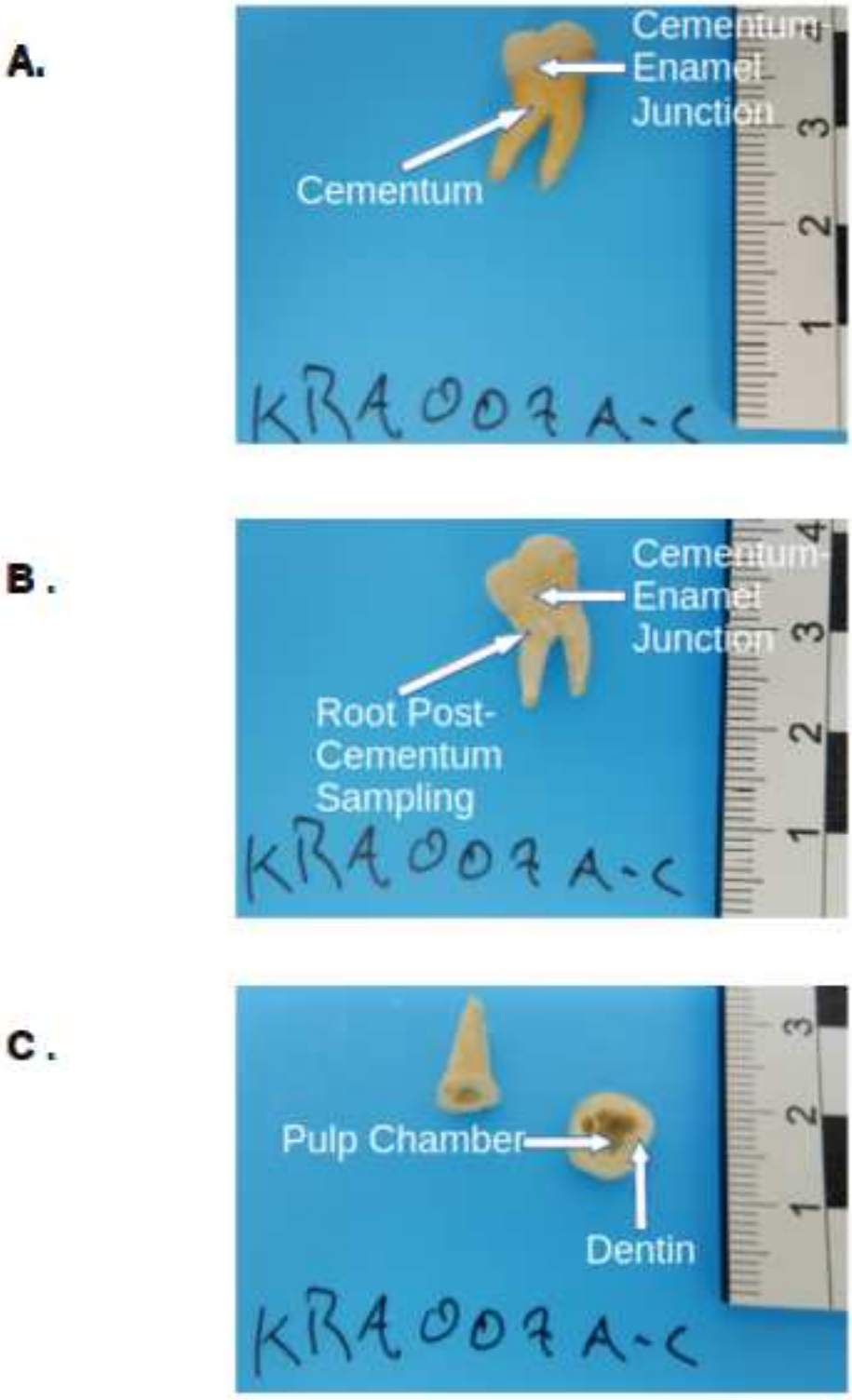

Figure 3

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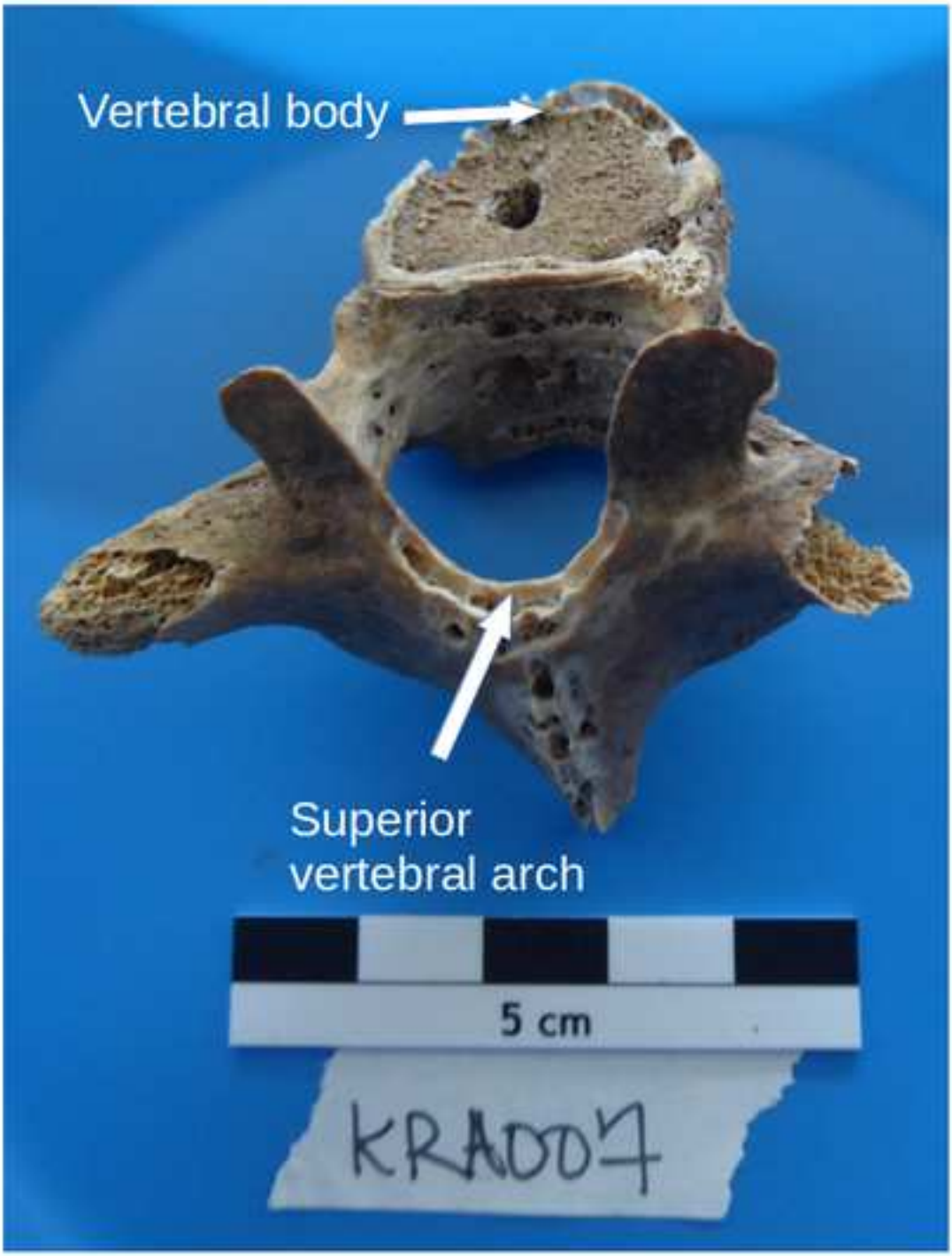

Figure 4

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Figure 5

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Figure 6

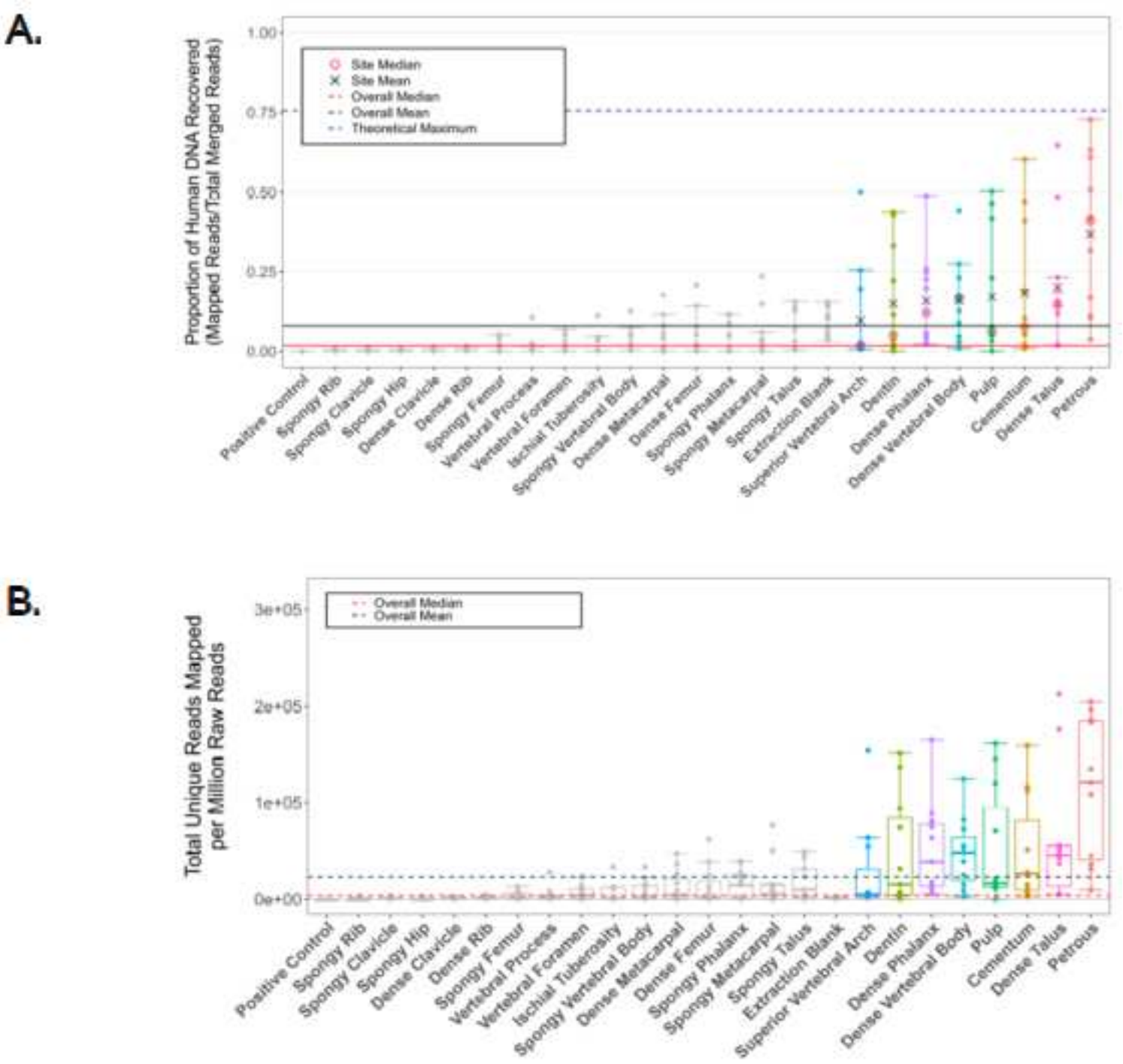

Table 1

Individual	Sex	Estimated age at death	^{14}C dates (CE, Cal 2-sigma)
KRA001	Male	25-35	1058-1219
KRA002	Female	20-22	1227-1283
KRA003	Male	25	1059-1223
KRA004	Male	15	1284-1392
KRA005	Male	10-12	1170-1258
KRA006	Female	30-40	1218-1266
KRA007	Female	25-30	1167-1251
KRA008	Male	20	1301-1402
KRA009	Male	Unknown	1158-1254
KRA010	Male	25	1276-1383
KRA011	Female	30-45	1040-1159

Sampling location	Average duplication factor (# mapped reads /# unique mapped reads)	Average fragment length in bp	Average estimated proportion of X chromosome contamination
Petrous pyramid	1.188 ± 0.006	65.40 ± 1.36	0.000 ± 0.003
Cementum	1.197 ± 0.028	67.28 ± 1.76	0.011 ± 0.003
Dentin	1.188 ± 0.061	60.22 ± 2.37	0.002 ± 0.007
Pulp	1.179 ± 0.024	55.14 ± 2.90	0.013 ± 0.006
Distal phalanx	1.191 ± 0.049	65.95 ± 1.08	0.013 ± 0.005
Vertebral body	1.194 ± 0.037	66.14 ± 1.03	0.008 ± 0.003
Superior vertebral arch	1.19 ± 0.017	63.02 ± 1.23	0.021 ± 0.009*
Talus	1.198 ± 0.010	68.20 ± 1.24	0.011 ± 0.003

*Sample KRA005 removed as an outlier at 0.1952

Table of materials

Click here to access/download

Table of Materials

JoVE_Materials_R2.xls

Reviewers and JoVE Editors,

Please find our point-by-point responses to your suggestions/concerns immediately following this letter. The format of our response is:

(1). Your comment.

Our response.

Additionally, among the uploaded files ,we have attached a revised version of the manuscript with the revisions highlighted in yellow. Areas highlighted in green are those protocols we propose for filming.

Thank you for your time and effort in reviewing this manuscript.

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done.

2. Please provide at least 6 keywords or phrases.

Done.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Done.

5. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Done.

6. Line 162-164: Please move the lines to the Figure and Table Legends section. If you want to include Figure 1 here, please mention “[Insert Figure 1 here].

Done.

7. Please highlight up to 3 pages of the Protocol (including headings and spacing). that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done.

8. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Done.

9. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Done.

10. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:

Done.

a). Critical steps within the protocol

b). Any modifications and troubleshooting of the technique

c). Any limitations of the technique

d). The significance with respect to existing methods

e). Any future applications of the technique

The discussion as a whole has been reorganized/revised to more clearly meet these criteria (Lines 482-514).

11. Please ensure that the Table of Materials includes all the essential (reagents, chemicals, consumables, surgical instruments, equipment, etc.). use in this study.

Done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Parker et al. have provided a protocol for sampling various bone elements that yield the greatest aDNA from human remains. It is clear, but some extra detail would be beneficial for first-time users to repeat their method exactly. A quick search on JOVE shows there isn't a similar protocol already and therefore it will be useful for many in the field of aDNA and forensic science (even potentially those not working on human remains). I recommend this protocol be published with minor revisions.

We thank the reviewer for their detailed and thorough comments, as we feel addressing the points raised has significantly improved the quality of the manuscript.

Major Concerns:

I don't have any major concerns, just suggestions for additional points that could be added to make the protocol more comprehensive (see below).; some might take a bit more effort to implement than others and can be addressed at the discretion of the editor as they may be beyond the scope of the journal (for example, the safety / waste disposal / ethics points made below).

Minor Concerns:

Keywords

(1). Line 24: It could be a good idea to include 'forensic' or something in the key words to make it more accessible to people in that field—I think it could be useful there too. Also a point for the discussion.

Thank you for your suggestion, we have added Forensics into the keywords accordingly (Line 25).

Summary

(2). Line 29, 'Medieval': Maybe include a time or age range here.

Agreed, we have revised to include the range of radio-carbon dates encompassing the samples (lines 42-43, and lines 73-74) as well as adding a table (reproduced from our original publication) to include this data as well as estimated age at death and sex of each individual (now Table 1, introduced in line 105).

Abstract

(3). Line 47: I think it would be useful to reference the article you're referring to here (your Scientific Reports paper).

Reference added (line 75).

(4). Line 49: Age range?

Updated accordingly see response to point 2.

Introduction

(5). Line 70: Probably 'specimen' is more appropriate word, to me 'artefact' refers to something non-biological made a by a human.

Changed accordingly (line 92).

(6). Line 76: Occasionally it's a bit confusing to see 'sampling locations' as in lots of papers that means the geographic area where the samples originated. Is there a less ambiguous term that could be used instead here (and throughout)? Not a big deal though.

We can appreciate how this could lead to confusion. As such, the terms "sampling location" and sampling site" have been replaced with "anatomical sampling location" throughout and a brief definition given on first usage (Lines 98-100).

(7). Line 79: Age range? I guess one limitation is that the method isn't compared to skeletons from different places or times so it may not be applicable for say skeletal remains recovered from the equator or remains that are tens of thousands of years old? You do discuss this but it's good for people to know how old things are from the get-go.

The manuscript has been revised to not only explicitly list the radiocarbon date ranges, but also to reference the new Table 1 that contains individual sample demographics such as estimated age at death and genetic sex. (Lines 104-105).

(8). Line 96: And possibly forensic science?

We thank the reviewer for pointing this out and feel that it significantly increases the importance and usability of the publication. (line 125).

Protocol

(9). Line 99: Are these guidelines published anywhere? A reference would be good. Or maybe a statement like "these guidelines are included/ detailed in the steps below"

These specific guidelines are not currently publicly available, however the section has been revised to urge researchers to ensure they are in compliance with the local/institutional guidelines (lines 130-137).

(10). Line 101: I think it's important to put a note about the ethics of this: what ethical approval or permissions are generally needed for this work? Maybe a statement like "ensure you are adhering to all local/state/federal ethical requirements for your area" …but beyond that (I don't want to sound to unscientific), maybe something about the fact that you should always be mindful that you are working on HUMAN remains. These were people, so everything should be treated with respect and dignity—they aren't just a sample or a specimen. People can forget this. In many cases there are living humans that still have a connection these remains so least destructive sampling /minimal harm is important for that aspect too. The researcher should try not to 'waste' anything and be mindful of how any 'waste' material is disposed of (from a safety point of view but also, you want to avoid treating a human's body like trash where possible).

See above

(11). Line 101: On that, are there any extra precautions people need to take when handling human remains specifically from a safety standpoint? If you are looking at pathogens, could the remains be infectious or hazardous (well, even if you aren't, could this person be harbouring a dormant infectious disease)? Or could the depositional environment contain soil pathogens? Is a special level of mask like N95 required over just a standard dust mask or surgical, which is often used? Dust masks also have a limited life-span so need frequent replacing. Inhalation of particulates of any kind can be hazardous but of humans in particular could this be more concerning for catching disease? Safety notes to this effect could be good to include.

(12). Line 103: If you are going to have caution statements throughout you might also want to have a statement about the typical time it takes to do each step with a disclaimer that you should take regular ergonomic breaks (especially in

a clean facility). like e.g., don't exceed 2hrs at time (or something) ? I don't know if you are required to have safe working instructions throughout but it could be a nice idea if this is going to be the go-to reference for people.

(13). Line 104: "Full personal protective equipment including clean …. should be worn. Certain PPE (such as gloves). should be frequently changed especially between samples. All equipment should be thoroughly decontaminated in between samples with … (bleach / DNAerase / ethanol) and UV irradiated where possible (e.g., drill bits, drills, pipettes, etc.)"

Points 10-13 have all been addressed in a newly added introduction to the protocol (Section 1, Considerations before sample processing, lines 128-155).

(14). Line 107: Why are they optional? What needs to be considered when deciding when to do this and when not to? e.g., would you not do this for a very old specimen? How does one decide?

The manuscript has been revised to clarify that the use of bleach and/or surface removal is a matter of some debate, as both may influence overall DNA yields (with references to a selection of appropriate publications testing these methods of pretreatment) and their use should be considered on a case-by-case basis (lines 162-168).

(15). Line 112, "dry sterile wipe": Lint-free too…

Updated accordingly. (line 177).

(16). Line 115: Can the bleach penetrate into the bone from the surface? Is it necessary if UV irradiating anyway? Could it even be detrimental? Perhaps a reference here would be good.

While this is a good caution, we feel as though the new sampling note to clarify why these protocols are considered optimal (lines 162-168). already sufficiently addresses this.

(17). Line 121: I think this should be "ultrapure, DNase/RNase free water", no?—there is no guarantee that deionized water is free from contaminants. This comment applies throughout. I don't know if this would impact things but the pH of some of these ultrapure waters is quite low and may impact the DNA?

The protocol has been updated throughout to refer to ultrapure water. (e.g., Line 181).

(18). Line 123: Ultraviolet—specify the wavelength. A caution note is also appropriate here as UV is very damaging to the skin and eyes; most institutions require radiation training to operate UV sources.

The protocol has been updated throughout to specify a wavelength of ca. 254 nm (e.g., line 171).

(19). Line 127, "cross-link in the presence of water": Reference?

Removed for simplicity's sake, as we understand this to be a fairly controversial viewpoint and the practice of UV decontamination is well documented in aDNA work.

(20). Line 130: Probably this note should be at the start because sometimes depending on the documentation level it needs to happen outside a clean environment. For instance, if you are having it micro-CT scanned, 3D imaged, etc. So there's no point decontaminating it then taking it out to get contaminated again. A heading such as "before you start" might be a good place to include things like this, the safety requirements, overall considerations, etc.

The pre-sampling considerations section has been updated to more strongly urge the proper documentation of samples BEFORE SAMPLING as well as to explicitly state that the protocols for doing so are NOT included in these steps (lines 151-155).

(21). Line 130: I think you could mention some suggestions for basic documentation / guidelines, or else provide a reference. E.g., accessioning in a database, having the accession in the photo (like you did., a scale, etc. Possibly even a colour square for white balancing. Weighing the specimen. Background considerations. I know digitising a specimen is its own protocol so not too much detail needed but I think it's an important part of the process that should people need to be aware of—directing to a reference would be enough or just flagging it to consider.

See above.

(22). Line 134: Where is this happening inside the facility? A dedicated room? On the bench? In a PCR hood / biological safety cabinet that can be UV'd? A fume hood (on/off)? Have you laid something down to catch powder (bench coat? alfoil?)? Has the bench been wiped over? Do you 'sterilise' the alfoil? See note above about dust powder being hazardous. Potentially drilling should occur in a space where dust can be contained and

decontaminated easily between samples. i.e., if it's put into the air on the bench then you may need to wait X amount of time for the dust to literally settle before decontaminating the bench…

The protocol has been revised throughout to state in the first step that all sampling should be performed in a UV capable biosafety cabinet located within a dedicated clean room and with its benchtop surface covered with sterile aluminum foil. The materials table has been updated and referenced here accordingly (e.g., lines 170-174).

(23). Line 140: Some things might be obvious to us but for a 'best practice' protocol I think certain details that you do without thinking need to be mentioned. Like make sure the clamp or vice is clean, and clean it between samples.

The protocol has been revised throughout to include a cleanup/decontamination step at the end of each protocol, specifically stating that cleanup should be done. between each sampling iteration (e.g., Lines 265-267).

(24). Line 142: What speed? Do you need to make sure it's fast to avoid cracking? Do you need to make sure it's slow so the sample doesn't heat up? How big are the teeth of the saw (this might not be 'standard' between countries)?

The step has been updated to clarify a 0.6mm blade and refer to the Materials table and caution regarding overheating of the sample (Lines 224-226 referencing lines 242-245).

Safety points about checking electrical equipment/having it safety tagged? Safety points about handling saws and cutting implements (might a first aid kit be required to be on hand? Should you be working completely alone/in isolation? should you not do this after-hours? Should you have access to a phone inside the aDNA facility? Do people know where you are? Do you need specific training for these?) Perhaps even just a note about having all risk assessments for tasks complete/ reviewed at regular intervals according to your institution's requirements. Same goes for the chemicals (store in corrosives cabinet according to guidelines, read the MSDS, ensure they are registered, have a chemical spill kit on hand, where is the safety shower, how can they be disposed, etc.).

The manuscript has been revised to urge researchers to refer to and follow their institution's safety standards in the process of performing all parts of this protocol (line 148).

(25). Line 146: Place on the electronic balance (accurate to what decimal place? is it the kind that has glass all around it or just an open scale?) and 'zero'.

The protocol has been revised to clarify the balance should be a hooded balance accurate to the 100th mg place and references the materials table throughout added throughout (e.g., lines 248-249).

(26). Line 150: Cross reference to photo?

Updated throughout the protocol (e.g., line 238).

(27). Line 150: Have you got a cat # for the drill bit used (or cross-refence here to materials table)? Not all are created equal. Also, these small gauge bits (especially if they aren't Dremel brand) require you to buy a smaller 'collet' for the Dremel. I ran into this problem once myself, thought I was good to go then the bit didn't fit snug into the Dremel because the collet was too big and I had to go order a custom one.

We agree that the exact brand/size/shape of bit used can vary quite widely, depending on the workers preferences. The bit (and set of collets. has been updated in the materials table, which has also been subsequently referenced throughout the paper (e.g., line 239).

(28). Line 151: Again, only relevant for a specific model of Dremel. What model did you use? (cross-reference to materials table).

See above

(29). Line 155: I wish, smells more like burnt hair to me, haha :\. But also, if you can smell it, might that indicate the PPE isn't working well enough?

The level 2 surgical masks indicated in the materials table are intended to block most particulate matter (98% at 3 um), as such smell sensitivity is reduced, however the ability to identify and classify smells is not (Chen et al 2020). and is, therefore, not likely to be an indication of PPE failure. The protocol has been updated to more clearly state that the smell is indicative of sample overheating (lines 242-245).

(30). Line 158: Space between number and units needed throughout. Also, what dictates 50 vs 100 mg (or anywhere in between)? Why WOULDN'T I just sample as close to 100 mg always? I know your paper has been peer reviewed so I won't analyse it too much, but if the extractions had different amounts of material, did you adjust for that in your calculations of yield? e.g., sure X site might give lower % human DNA but only X mg was extracted. Or the opposite (it had the same % DNA but so much less was extracted making it preferable? Could be a good discussion point)

A note has been added to clarify why 100 mg may be preferable to 50 mg (as it allows for two extractions). when available, and also states that conversely this may not always be possible based on the element selected (e.g., cementum) or the need for morphological preservation. It is also now stated that those anatomical sampling locations regularly yielding less than 50 mg have still been assessed to harbour comparable, if not better, endogenous DNA than many samples of much greater size and are thus still desirable based on a direct extraction vs extraction yield basis (lines 251-257).

(31). Line 158: Use full stops at the end of each point for consistency.

Thank you for finding this, the protocol has been updated throughout.

(32). Line 160: low-bind, Safelock Eppendorf tube (or brand whatever). Specify the lid because it's important that it's screw-cap or Safelock for the subsequent DNA extraction. You don't want to have to be transferring powder between tubes and end up losing some.

The protocol has been revised throughout to specify safe-lock tubes and the materials table updated accordingly (e.g., line 259).

(33). Line 160: Storage? Proceed immediately to extraction? Put it in the 4 deg C fridge? Put it in 12 deg C (wine) fridge? Leave at RT? Freeze it at -20 deg C? Freeze it at -80 deg C? How should we store it until we are ready to extract it? Why or why not?

The protocol has been revised throughout to recommend storage of fresh bone powder at -20 C before extraction (e.g., lines 259-260).

(34). Line 163: Could this not be drilled without cutting it in half? Would this be considered destructive?

The manuscript has been revised to more clearly state that less destructive protocols for the pars petrosa are now available (Sirak et al 2017 and Orfanou et al 2020 in particular) and to more clearly point the readers towards them for use with samples where morphological preservation is highly suggested (lines 210-214).

(35). Line 166: How are we disposing of the waste? Waste weigh boats and paper would be considered biological waste and may need to be autoclaved prior to incineration (and Kim wipes, paper towel, etc.) So they should be disposed of in an autoclavable biological hazard bag. Are we cleaning drill bits /saw blades for reuse or throwing them out? What's the cleaning process? If throwing away they would potentially need to go in a sharps bin. Again, this would be a good note to include in a "before starting" section.

This is an excellent suggestion and the protocol has been revised throughout in order to include a specific disposal step for all waste generated during the sampling of any element, as it may be contaminated with human remains (e.g., Lines 265-267).

(36). Line 172: cavities you mean?

Yes, although it is our understanding that the term "caries" is a bit more inclusive as it includes areas of tooth decay that have not yet developed into holes (cavities) in the enamel.

(37). Line 172: Or excessive dental calculus? Could that be a source of contamination (if you aren't interested in the microbiome of course) that should be drilled off and discarded or avoided?

The note has been amended to include a statement that calculus should be removed and stored for independent analyses (lines 274-276).

(38). Line 174: How do you decide if this is appropriate or not? How cold? Freeze-thaw can also be harmful for DNA…

As this is a purely optional step based on personal preferences in pathogen, not human DNA retrieval, it has been removed from the protocol.

(39). Line 179: Same comment as above applies throughout.

See above

(40). Line 187: Root? (room is written).

Thank you for the correction, root was the intended word (line 288).

(41). Line 202: Refer to photo

Done. (line 313).

(42). Line 208: Cross reference to photo

The protocol has been revised throughout to cross reference the mention of anatomical sampling locations with the appropriate figure (e.g., lines 224, 238, 313, etc.).

(43). Line 245: This note should come at the start of the protocol or after ever optional surface removal step.

Agreed, we have included the possibility that surface removal may be deleterious to DNA recovery, including citation into the pre-sampling considerations (lines 162-168, 387, 422).

(44). Line 316: Put note before 2.5.1

Done.

(45). Line 319: I think you should have a little section about 'after care' as well. Where do you store any left-over material? Where do you store left over bone powder long-term? Does bone powder need to be treated as biological waste? Also, a note about potentially repatriating remains—some people may even want left-over bone powder repatriated…Do you need to put remains in a museum? Or a biobank?

The protocol has been revised throughout to include a storage step (for the purposes of repatriation/return). of excess bone and bone powder. We believe that all excess bone AND powder should be returned to its proper owner as soon as possible after sampling (e.g., lines 262).

(46). Line 319: What controls can you suggest during the sampling stage? Does the researcher need to sequence their own DNA? Should you include any negative controls here? Positive controls? Controls for the room (air, surface controls)? Should you perform replicates? How many?

The experimental design of any study using these protocols falls outside of the scope of this manuscript and we do not feel that we can comprehensively cover the myriad possibilities here. However, we feel it important to note that we do agree that all of the above are needed in order to adequately assess the amount of contamination which may be present in any given sample set.

Representative Results

(47). Line 321, "in a separate study": Reference needed.

Noted and revised (line 496).

(48). Line 334: But if we had a choice of only one of these other locations, which would we pick? what gives the best value in terms of least amount of material needed, least impact on the appearance of the specimen, best yield? Or even if it's not the best yield it's sufficient and least destructive?

Added a concluding sentence recommending the teeth and vertebrae specifically as they each have multiple viable anatomical sampling locations (lines 522-524).

(49) Line 352: I do find this confusing, why not just plot the % of unique mapped reads out of the total unique reads? (or even if you do rarify it out of 1 million for computational ease, still why not present it as a %?) Or, if you are showing complexity, show % total unique out of total raw?

The method calculation of endogenous DNA here is used as it is the default measure used by the EAGER pipeline, and as such more accurately represents the measure expected to be observed if replicated using the same pipeline. Our second measure (unique reads per million reads sequenced. is used for both computational ease and is

interchangeable with a % measure of the same. We have used these measurements in this manuscript to maintain continuity with the original publication (Parker et al 2020. for ease of reference.

(50). Line 353: what do the error bars represent here? Also don't really understand why it's 'predicted' —can't you just plot what you DID recover?

This analysis was a part of the original publication using these protocols (Parker et al 2020., we agree that it is superfluous here and have removed it from the figure/text.

(51). Line 355: 55,000? You've got 55,0000 written

See above

Discussion

(52). Line 367: As shown here, right? wouldn't this be considered destructive? Or no?

The manuscript has been revised here (and in the introduction) to reflect that newer, much less invasive techniques are now in practice (e.g., Sirak et al 2017. and should be considered first for samples where morphological preservation is absolutely necessary (lines 210-214, 537, 551).

(53). Line 372: Do all these sites just have one element per human? could it make it tricky to distingish between individuals otherwise? could be an idea to also suggest sampling more than one location where possible as a replicate? That may help distinguish contamination—might be an idea as a control?

The manuscript has been revised to incorporate this potential limitation/solution (lines 555-566).

(54). Line 382: Mention the host thing like you did in your paper—that if you want microbiome or pathogens, other methods might be preferable. Could also suggest that maybe the sampling method, especially for teeth, could be applicable to non-human mammals?

This too has now been incorporated into the limitations of these protocols, with the caveat that further studies into all of these contexts are necessary (lines 555-566).

Table 1

(55). If you're going to have this table, why not include the confidence intervals for these values as well?

Added corresponding standard error of the mean to all means, additionally have included a supplemental file of all relevant raw data in xlxs form.

(56). The cluster factor is a bit confusing, why not just show %?

Cluster factor is the ratio of unique mapped reads to all mapped reads and as such indicates the expected number of duplicates per unique read. Additionally, this is again the default measure used in the EAGER pipeline, and thus presented here to maintain continuity with the other results presented using this pipeline.

(57). You mean contaminating human DNA not just 'non-endogenous'? % would be easier to interpret in my opinion. In the discussion you could talk about how dentin and vertebral body have much lower rates of contamination so even if they don't yield as high DNA as some of the others they could be preferable? Or has this already been factored in?

There is no statistical difference between the contamination estimates of any of these anatomical sampling locations, thus we cannot state that any location is better than any other in this case. They have been presented in proportion rather than percentage to more intuitively reflect this.

Table of materials

(58). The clamp or vice grips? The UV steriliser? Electronic balance? Ethanol? Do you need to mention PPE? Bench coat? Sharps bin / biological hazard bin? Camera, scale?

While the camera and scale are beyond the scope of these protocols, the Materials Table has been revised to include examples of all PPE, biological hazard bags, aluminum foil, and hood, etc..

Reviewer #2:

Manuscript Summary:

This work can become a useful guideline for several researchers with no previous experience of sampling skeletal material for aDNA analysis. It provides sampling protocols for several key skeletal elements and anatomical sites. Overall, the protocols are clear and succinct. Therefore, I believe that it is worth of publication.

We thank the reviewer for their helpful comments and suggestions and sincerely hope we have alleviated their concerns.

Major Concerns:

-I don't agree with the use of bleach. Bleach can change the structural and chemical characteristics of the specimen to an unknown extent since the macro-, micro-, and nano-porosity can significantly vary depending on the skeletal element and its preservation state (penetration depth) I believe this part should be deleted as it can affect the data of other analytical methods.

We recognize the use of bleach or surface removal for decontamination is highly debated, as such we have revised the pre-sampling considerations to more clearly indicate this, as well as added appropriate references. (Lines 162-168).

Minor Concerns:

-Although it has been mentioned in the discussion, I don't believe that sampling the petrous bone like that is the optimum way. Actually it's quite destructive. While you mention that there can be other ways of doing this, from the moment this protocol will be published many researchers may stick to it. And since you collect bone powder by drilling, there are other ways to do that without cutting the petrous bone in half.

The current, less invasive, sampling methods (Sirak et al 2017) were not available when the supporting analyses/publication was done. That being said, we still feel that this method is considerably easier for the novice sampler. In order to clarify this we have revised the manuscript to more clearly state that less destructive alternatives are available and to more clearly point researchers towards them/urge the consideration of their use for specimens where morphological preservation is of utmost importance (lines 210-214, 537, 551).

Reviewer #3:

Manuscript Summary:

This paper describes an updated sampling protocol for skeletal elements for the retrieval of ancient human DNA. The authors present one traditional sampling location (pars petrosa) and protocols on an additional seven sampling sites. The goal is to allow maximum morphological preservation but have optimal sampling yields. The authors present the full protocol used for each sampling site and present data reflecting the overall yield from each site/bone to illustrate their average yield relative to the pars petrosa more traditionally sampled. The authors found that the pars petrosa continued to have the highest average yield but that the other four elements sampled showed comparable yields to one another and produced sufficient sequence profiles.

We thank the reviewer for their helpful comments and suggestions and sincerely hope we have alleviated their concerns.

Major Concerns:

None.

Minor Concerns:

The experimental design presented in the abstract and introduction discusses the 11 individuals that had skeletal elements sampled across five skeletal elements. However, in the introduction (line 83) the authors state seven sampling locations (instead of 8) across five skeletal elements instead of 4. It seems implied that the eight and fifth element/sample is the pars petrosa, but the authors need to provide some clarity. Are they including the pars petrosa sample in their experimental design or is it the baseline for comparison. This should be stated somewhere in the text. Further, in the representative results, the authors state that the pars petrosa yielded higher endogenous DNA than 23

other sampling locations (lines 328-331) I can see that they show 23 sampling sites in Figure 6, however, there is no accounting for this in the current paper. If this is from a previous study, that needs to be clarified and the study should be cited here. If only focusing on the 5 elements tested here, then figure 6 should be updated. Currently, there is no context for this result. This is a good protocol for this journal media but additional clarification for the representative data should be provided in the relevant sections.

The introduction (line 110-125) has been clarified to state that all eight anatomical sampling locations, including the pars petrosa are part of the original experimental design, with the supporting paper now referenced in line 114. Additionally, the representative results have been revised to earlier reference the supporting paper as well as clarifying that the protocols presented here are those used in the original study, a comparison of 23 separate anatomical sampling locations, which determined that these eight anatomical sampling locations performed best (lines 110-125, 505-509).

Reviewer #4:

Manuscript Summary:

The article describes protocols to collect bone powder from ancient human skeletal remains, to minimise sample size, preserve material, and maximise DNA yield.

We thank the reviewer for their helpful comments and suggestions and sincerely hope we have alleviated their concerns.

Major Concerns:

The article is clearly written and the descriptions are thorough. I have no concerns.

Minor Concerns:

Some of the sentences are long and cumbersome and some minor editing is recommended. In addition, terms such as "sampling locations" are ambiguous. The authors are referring to places on the bones, not the archaeological sites. I had to read the text twice to see that the term refers to different parts of a bone. Again, minor editing should help make the text more readable. But overall this is a clear, well written paper, which would be of interest to many researchers in aDNA.

The manuscript has been edited throughout to simplify sentence structure as well as to more clearly define the terms used, as such "sampling location" and sampling site" have been replaced with "anatomical sampling location" throughout and a brief definition given at first use. Additionally, the text has been edited throughout for simplicity.

TITLE:
Optimized bone sampling protocols for the retrieval of ancient DNA from archaeological remains

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Archaeogenetics, aDNA, Bone Sampling, Forensics, DNA Sampling, Bioanthropology

Summary:

Presented here are a series of best practice protocols for collection of bone powder from eight recommended anatomical sampling locations (specific locations on a given skeletal element) across five different skeletal elements from medieval individuals (radiocarbon dated to a period of ca. 1040-1400 CE, calibrated 2-sigma range). Prior research has suggested that these protocols maximize the chances for the successful recovery of ancient human and pathogen DNA from archaeological remains.

Abstract:

The methods presented here seek to maximize the chances for the recovery of human DNA from ancient archaeological remains while limiting sample input material by targeting anatomical sampling locations previously determined to yield the highest amounts of ancient DNA (aDNA) in a comparative analysis of DNA recovery across the skeleton. DNA yields were previously assessed in a broad survey of aDNA preservation across multiple skeletal elements from 11 individuals recovered from the medieval (radiocarbon dated to a period of ca. 1040-1400 CE, calibrated 2-sigma range) graveyard at Krakauer Berg, an abandoned medieval settlement near Peißen Germany (see Parker, C. *et al*. A systematic investigation of human DNA preservation in medieval skeletons. Scientific Reports. 10 (1), 18225, doi: 10.1038/s41598-020-75163-w (2020). These eight sampling spots, which span five skeletal elements (*pars petrosa*, permanent molars, thoracic vertebra, distal phalanx, and talus) all successfully yielded high quality ancient human DNA, where yields were significantly greater than the overall average across all elements and individuals. Yields were adequate for use in most common downstream population genetic analyses. Our results support preferential use of these anatomical sampling locations for most studies involving the analyses of ancient human DNA from archaeological remains. Implementation of these methods will help to minimize the destruction of precious archaeological specimens.

Introduction:

The sampling of ancient human remains for the purposes of DNA recovery and analysis is inherently destructive[1–4]. The samples themselves are precious specimens and morphological preservation should be preserved wherever possible. As such, it is imperative that sampling practices be optimized to both avoid unnecessary destruction of irreplaceable material and to maximize the probability of success. Current best practice techniques are based on a small cohort of studies limited to either forensic surveys[5, 6], studies of ancient specimens where the development of optimal sampling is not the direct aim of the study[7], or dedicated studies utilizing either non-human remains[8] or targeting a very small selection of anatomical sampling locations (used here to denote a specific area of a skeletal element from which bone powder for use in downstream DNA analyses was generated)[9, 10]. The sampling protocols presented here were optimized in the first large scale systematic study of DNA preservation across multiple skeletal elements from multiple individuals[11]. All samples stemmed from skeletal elements recovered from 11 individuals excavated from the church graveyard of the abandoned medieval settlement of Krakauer Berg near Peißen, Saxony-Anhalt, Germany (see Table 1 for detailed sample demographics) and, as such, may need modification for use with samples outside of this geographical/temporal range.

[Insert Table 1 here]

These protocols allow for the relatively straightforward and efficient generation of bone powder from eight anatomical sampling locations across five skeletal elements with limited laboratory-induced DNA contamination (including the *pars petrosa*). Of these five skeletal elements, seven

anatomical sampling locations found on four skeletal elements have been determined to be viable alternatives to the destructive sampling of the petrous pyramid[11,12]. These include: the cementum, dentin, and pulp chamber of permanent molars; cortical bone gathered from the superior vertebral notch as well as from the body of thoracic vertebrae; cortical bone stemming from the inferior surface of the apical tuft and shaft of the distal phalanges; and the dense cortical bone along the exterior portion of the tali. While there are several widely applied methods for the sampling of the *pars petrosa*[4,12-14], dentin, and the dental pulp chamber[1, 2,15], published methods describing the successful generation of bone powder from the cementum[16], vertebral body, inferior vertebral notch, and talus can be difficult to obtain. As such, here we demonstrate optimized sampling protocols for the petrous pyramid (Section 2.1); cementum (Section 2.2.1), dentin (Section 2.2.2), and dental pulp (Section 2.2.3) of adult molars; cortical bone of the vertebral body (Section 2.3.1) and superior vertebral arch (Section 2.3.2); the distal phalanx (Section 2.4); and the talus (Section 2.5) in order to make the effective use of these skeletal elements for both aDNA and forensic research more widely accessible.

Protocol:

1. Considerations before sample processing:

All research presented herein was performed in compliance with the guidelines set forth by the Max Planck Institute for the Science of Human History, Jena, Germany for working with ancient human remains. Before performing any steps of this protocol ensure to adhere to all local/state/federal ethical requirements pertaining to both obtaining permission for the scientific study and use of human remains for destructive sampling in your area. Additionally, as ancient

remains are an irreplicable and finite resource it is imperative that all samples be treated accordingly (*e.g.*, sampling should be as minimally wasteful as possible, and all remains returned to their respective and lawful providers if possible).

All following steps should be performed in a clean-room environment, preferably at a dedicated ancient DNA facility[17-19], using personal protective equipment (PPE) consisting of sterile microporous coveralls with hood, sterile gloves (two pairs), surgical mask, protective eyewear, and sterile boots or non-slip shoes with sterile covers (see Materials Table for examples). It is recommended that gloves be changed frequently, especially between samples. Additionally, all equipment and surfaces should be cleaned and disinfected thoroughly with bleach/DNAerase/ethanol and UV irradiated (wavelength: ca. 254 nm) where possible (*e.g.*, drill bits, drills, vices/clamps, *etc.*). Finally, it is highly recommended that regular ergonomic breaks be taken (every 2-3 hours if possible) to avoid over-exhaustion due to the clean-room environment. All procedures/chemical storage should be performed according to individual institutional safety guidelines.

NOTE: All skeletal remains should be appropriately documented (*e.g.*, photographed, weighed, and if possible micro-CT scanned, 3d imaged, *etc.*) before sampling (protocols for appropriate documentation are not covered in this manuscript). All sampling protocols may be paused between sampling iterations and the samples stored indefinitely in a dry, temperature controlled (25 °C), sterile environment.

2. Pretreatment

2.1 To minimize the risk of contamination it may be advisable to decontaminate all anatomical sampling locations prior to bone powder generation[18].

NOTE: The efficacy of bleach and/or surface removal for sample decontamination is still a matter of debate among aDNA researchers[8,19-25] as both may influence overall DNA yields, especially in highly degraded samples. As such, the following steps are considered optional, and are included here as they were used in all samples to generate the representative results presented in this paper. It is recommended that the use of these pre-treatment protocols be determined on a case-by-case basis based on the molecular application, age, rarity, and levels of morphological degradation of each sample set.

2.1.1 Perform all sampling in a dedicated clean room. In a UV capable PCR hood or biosafety cabinet (UV wavelength: ca. 254 nm) with airflow turned off spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments. Ensure all bone fragments are recovered (for repatriation) before disposing of foil. Change foil between the treatment of each skeletal element. Dispose of used foil in an autoclavable biohazard bag/receptacle.

2.1.2 Remove as much loose dirt/detritus as possible from anatomical sampling locations by gently wiping the area with a lint-free dry sterile wipe (see Materials Table for example). Dispose of wipes in autoclavable biohazard bags or receptacles.

2.1.3 Decontaminate the cleaned surface by wiping with a sterile wipe moistened with diluted commercial bleach (~0.01% v/v, diluted with ultrapure DNase/RNase free water) and allow to incubate for 5 minutes. Dispose of wipes in autoclavable biohazard bags or receptacles.

CAUTION: Bleach is a highly corrosive and reactive chemical; hence appropriate safety precautions should be in place before its use.

2.1.4 Remove as much residual bleach as possible from the anatomical sampling location with a sterile wipe moistened with ultrapure DNase/RNase free water. Dispose of wipes in autoclavable biohazard bags or receptacles.

2.1.5 Expose all cleaned anatomical sampling locations to U/V radiation for 30 minutes (wavelength ca. 254 nm), then allow to dry fully at room temperature.

CAUTION: Exposure to UV radiation can be harmful to the eyes.

2.1.6 Move immediately to sampling or store skeletal elements in a dry, temperature controlled (25 °C) sterile environment.

CRITICAL STEP: It is crucial that anatomical sampling locations be completely dry before proceeding with sampling or returning to storage to not only make bone powder generation easier, but also to prevent further degradation of the sample (*e.g.*, mold).

3. Bone powder generation

NOTE: All following protocols are intended for use in DNA extraction following the Dabney *et al* 2019 protocol[26].

3.1 Sampling of the *pars petrosa*

NOTE: This protocol is adapted from procedures described in Pinhasi *et al*, 2019[4] and is presented here for ease of use. This protocol does not represent the current, least destructive method for the sampling of *pars petrosa*. As such, it is recommended to use the protocol described by Sirak *et al* 2017[13] or Orfanou *et al* 2020[14] for samples where morphological preservation is of maximum import.

3.1.1 Perform all sampling in a dedicated clean room. In a UV capable PCR hood or biosafety cabinet (UV wavelength: ca. 254 nm) with airflow turned off. Spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments. Ensure all bone fragments and as much powder as possible is recovered (for repatriation) before disposing of foil. Change foil between each sampling. Dispose of used foil in an autoclavable biohazard bag/receptacle.

3.1.2 Secure the dry, decontaminated element using a sterilized clamp or vice.

3.1.3 Cut the *pars petrosa* in half along the superior *sulcus petrosus* (see Figure 1) using a standard jeweler's saw equipped with a 0.6mm blade (see Materials Table) at medium speed to avoid overheating (see CRITICAL STEP for this section of the protocol).

CAUTION: The *pars petrosa* is very dense, and as such may be difficult to cut. As such, take care to keep the element securely clamped to avoid injury. Dispose of any broken saw blades in an appropriate sharps' receptacle.

3.1.4 Remove the petrous portions from the clamp and remove any loose material.

3.1.5 Place weigh paper into a sterile weighing boat

3.1.6 Hold petrous portion over weigh paper, cut side tilted towards weighing tray. Drill into the dense cortical bone between the facial canal and mastoid antrum (appears "shinier" than surrounding material, see Figure 1) using dental drill/Dremel equipped with a small gauge bit (see Materials table for example) set to medium speed, medium torque to avoid overheating while still providing enough traction for bit to gain purchase.

CRITICAL STEP: Drilling/Cutting should be done in short bursts to avoid overheating the bone and potentially destroying/damaging DNA. Anecdotally, when the dense portion of the petrous begins to overheat a smell described as "cooking bacon" may be observed. Cease drilling/sawing immediately and allow the bone to rest until sufficiently cool before resuming.

3.1.5 Repeat drilling until approximately 50-100 mg of powder are collected in the weigh paper, as measured using an enclosed balance accurate to at least the 100th mg (see Materials Table for example).

NOTE: Where possible it is suggested to gather ca. 100 mg of bone powder to allow for two replicate DNA extractions of 50 mg each. However, this may not always be possible based on either limitations of the anatomical sampling locations themselves (*e.g.*, the distal phalanx, dental pulp chamber) or need for morphological preservation. For other locations, such as the cementum, considerably less than 50 mg of material may be available. However, the cementum, dental pulp chamber, and distal phalanx have all been shown to yield significant endogenous DNA[11,27,28], despite lower initial input of bone powder into the extraction process.

3.1.6 Transfer powder from weigh paper to a 2 ml labelled low-bind, safe-lock tube for extraction or storage. Store at (-20 °C) indefinitely.

3.1.7 Store remaining bone/excess powder in a dry, temperature controlled (25 °C) sterile environment until return/repatriation can be completed.

3.1.8 Dispose of all waste in autoclavable biohazard bags or receptacles. Sterilize/decontaminate all reusable equipment (*e.g.*, clamps, drill bits, drills, saws, *etc*.) using bleach/DNAerase/Ethanol and UV (wavelength: ca. 254 nm) exposure, as applicable, between each sampling.

[Insert Figure 1 here]

3.2 Sampling of permanent molars

NOTE: For the sampling of permanent molars, pre-select in-situ molars with fused roots and ideally void of caries, cracks in the enamel or excessive wear for best results. Remove any dental calculus before sampling and store at (-20 °C) for possible future analyses of the oral microbiome (procedure not covered here).

3.2.1 Cementum sampling

3.2.1.1 Perform all sampling in a dedicated clean room. In a UV capable PCR hood or biosafety cabinet (UV wavelength: ca. 254 nm) with airflow turned off spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments. Ensure all bone fragments and as much powder as possible is recovered (for repatriation) before disposing of foil. Change foil between each sampling. Dispose of used foil in an autoclavable biohazard bag/receptacle.

3.2.1.2 Place a sheet of weigh paper into a sterile weighing tray.

3.2.1.3 Hold/secure the decontaminated molar by the enamel, root down, over a weighing tray using a hand-held clamp such as an adjustable wrench (see Materials table for example).

3.2.1.3 Equip a Dremel/dental drill with a diamond edged circular cutting wheel. With the drill set to a medium speed/torque setting lightly touch the edge of the bit to the root at an

approximately -20° angle. Scrape downwards into the tray to remove/collect the yellow, outermost material from the root (cementum). Stop collection when the lighter (white) material of the dentin becomes visible.

CRITICAL STEP: It is important to match the direction of rotation of the cutting bit in relation to the collection tray to avoid the powder becoming aerosolized and potentially wasting sample by missing the tray entirely.

NOTE: The cementum is particularly rich in DNA, however typical yields of material are much smaller than other anatomical sampling locations (~7-20 mg)[11,27,28].

3.2.1.5 Record weight of powder collected in weigh paper using an enclosed balance accurate to at least the 100th mg (see Materials Table for example).

3.2.1.6 Transfer powder from weigh paper to 2ml low-bind, safe lock tube for extraction. Store at (-20 °C) indefinitely.

3.2.2 Sampling of the Pulp Chamber (Pulp and Dentin)

3.2.2.1 After cementum has been collected (if desired), section the molar along the cemento-enamel junction using a jeweler's saw to remove the crown (see Figure 2).

3.2.2.1 Place a new sheet of weigh paper in a new weighing tray.

3.2.2.2 Secure crown section in a handheld clamp or vice, over weighing tray. Hold cut side tilted downward and drill/scrape material as a first pass with a dental drill/Dremel equipped with a small gauge drilling bit (see Materials Table for example) along the edges of the pulp chamber within the crown portion (see Figure 2).

NOTE: Only the first pass of the interior of the pulp chamber is to be collected and labelled as "pulp" material (5-15 mg typical yield), anything deeper into the tooth is considered dentin.

3.2.2.3 Turn the tooth with the inferior portion facing down, tap the clamp with a hammer, and collect the liberated powder on weigh paper.

3.2.2.4 Record weight of powder collected in the weigh paper using an enclosed balance accurate to at least the 100th mg (see Materials Table for example).

3.2.2.5 Transfer powder from weigh paper to 2ml low-bind, safe-lock tube for extraction. Store at (-20 °C) indefinitely.

3.2.3 Sampling of the remaining dentin

3.2.3.1 Place a new sheet of weigh paper in a new weighing tray.

3.2.3.2 Hold crown section over weighing tray (as per 3.2.2.2), drill out and collect a further 50-100 mg of dentin as measured using an enclosed balance accurate to the 100th mg (see Materials Table for example) from the interior of the pulp chamber in the same manner for further dentin sampling (see Figure 2).

3.2.2.4 Transfer bone powder from weigh paper to 2ml low-bind, safe-lock tube for extraction. Store at (-20 °C) indefinitely.

3.2.2.5 Store remaining tooth pieces/excess powder in a dry, temperature controlled (25 °C) sterile environment until return/repatriation can be completed.

3.2.2.6 Dispose of all waste in autoclavable biohazard bags or receptacles. Sterilize/decontaminate all reusable equipment (*e.g.*, clamps, drill bits, drills, saws, *etc*.) using bleach/DNAerase/Ethanol and UV (wavelength: ca. 254 nm) exposures applicable, between each sampling.

[Insert Figure 2 here].

3.3 Sampling of the Thoracic Vertebrae

3.3.1 Sampling of the vertebral body

3.3.1.1 Perform all sampling in a dedicated clean room. In a UV capable PCR hood or biosafety cabinet (UV wavelength: ca. 254 nm) with airflow turned off spread sterile aluminum foil across

the benchtop to catch any stray bone powder/fragments. Ensure all bone fragments and as much powder as possible is recovered (for repatriation) before disposing of foil. Change foil between each sampling. Dispose of used foil in an autoclavable biohazard bag/receptacle.

3.3.1.2 Place a small sheet of weigh paper into a standard weighing tray.

3.3.1.3 Secure vertebrae with clamp or hand vice, vertebral body outward.

3.3.1.4 Hold vertebrae over weighting tray, vertebral body tilted downward. Using a dental drill/Dremel equipped with a small gauge drilling bit (see Materials Table for example) set to low-speed high torque, drill along the outermost rim (inferior and superior) of cortical bone surrounding the cancellous inner tissue of the vertebral body (see Figure 3), by scraping the bit against the cortical layer over a standard weighting tray until 50-100 mg of material as measured using an enclosed balance accurate to the 100th mg (see Materials Table for example) has been collected.

3.3.1.5 Transfer bone powder from weigh paper to 2ml low-bind, safe lock tube for extraction. Store at (-20 °C) indefinitely.

3.3.2 Sampling of the superior vertebral arch

OPTIONAL: Remove and discard the outermost layer of the cortical bone of the superior vertebral arch using a dental drill equipped with a small gauge drilling bit (see Materials Table

for example) by scraping it along the surface[19]. This is not suggested for sampling from the vertebral body, as the layer of cortical bone is generally very thin and likely to be entirely depleted by this process (See NOTE in Section 2. Pretreatment for cautions).

3.3.2.1 Place a small sheet of weigh paper into a standard weighing tray.

3.3.2.2 Secure vertebrae in hand clamp/vice with vertebral process outward, superior aspect down.

3.3.2.3. While holding vertebrae, superior aspect down, over a weighing tray, drill upwards into the center of the "V" shaped notch formed by the fusion of the spinous process with the lamellae (see Figure 3) using a dental drill/Dremel with small gauge bit (see Materials Table for example) set to low speed and high torque. Cease drilling when there is a noticeable drop in resistance. Change drilling position slightly and repeat until 50-100 mg of bone powder as measured using an enclosed balance accurate to the 100th mg (see Materials Table for example) has been collected.

3.3.2.4 Transfer bone powder from weigh paper to 2ml low-bind tube for extraction. Store at (-20 °C) indefinitely.

3.3.2.5 Store remaining bone/excess powder in a dry, temperature controlled (25 °C) sterile environment until return/repatriation.

3.3.2.6 Dispose of all waste in autoclavable biohazard bags or receptacles. Sterilize/decontaminate all reusable equipment (*e.g.*, clamps, drill bits, drills, saws, *etc.*) using bleach/DNAerase/Ethanol and UV (wavelength: ca. 254 nm) exposure, as applicable, between each sampling.

[Insert Figure 3 here].

3.4 Sampling of the Distal Phalanx

OPTIONAL: Remove and discard the outermost layer of the cortical bone of the shaft and/or apical tuft using a dental drill equipped with a small gauge drilling bit by scraping it along the surface[19]. This may not be possible for samples with excessively thin cortical bone or juvenile remains (See NOTE in Section 2. Pretreatment for cautions).

3.4.1 Perform all sampling in a dedicated clean room. In a UV capable PCR hood or biosafety cabinet (UV wavelength: ca. 254 nm) with airflow turned off spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments. Ensure all bone fragments and as much powder as possible is recovered (for repatriation) before disposing of foil. Change foil between each sampling. Dispose of used foil in an autoclavable biohazard bag/receptacle.

3.4.2 Place a small sheet of weigh paper into a standard weighing tray.

3.4.3 Secure sample in handheld clamp/vice superior side upwards.

3.4.4 While holding sample over weigh tray, collect bone powder from the cortical bone from the inferior side of the apical tuft and shaft by drilling through the outermost dense layers (see Figure 4) using a dental drill/Dremel with small gauge drilling bit (see Materials Table for example). Drilling should cease when there is a marked decrease in resistance, as this signifies lighter, cancellous material. Repeat this process, radiating outward from the initial drilling until at least 50-100 mg of bone powder as measured using an enclosed balance accurate to the 100th mg (see Materials Table for example) has been collected on the weigh paper.

3.4.5 Transfer bone powder from weigh paper to 2ml low-bind, safe-lock tube for extraction. Store at (-20 °C) indefinitely.

3.4.6 Store remaining bone/excess powder in a dry, temperature controlled (25 °C) sterile environment until return/repatriation.

3.4.7 Dispose of all waste in autoclavable biohazard bags or receptacles. Sterilize/decontaminate all reusable equipment (*e.g.*, clamps, drill bits, drills, saws, *etc.*) using bleach/DNAerase/Ethanol and UV exposure, as applicable, between each sampling.

NOTE: For smaller samples (*e.g.*, juvenile samples) there may be considerably less than the suggested 50-100 mg of cortical bone available to sample. However, even in low quantities this anatomical sampling location has been shown to be particularly rich in DNA[11].

[Insert Figure 4 here].

3.5 Sampling of the Talus

3.5.1 Perform all sampling in a dedicated clean room. In a UV capable PCR hood or biosafety cabinet (UV wavelength: ca. 254 nm) with airflow turned off spread sterile aluminum foil across the benchtop to catch any stray bone powder/fragments. Ensure all bone fragments and as much powder as possible is recovered (for repatriation) before disposing of foil. Change foil between each sampling. Dispose of used foil in an autoclavable biohazard bag/receptacle.

3.5.2 Place a small sheet of weigh paper into a standard weighing tray.

3.5.3 Secure sample in handheld clamp/vice, dome upwards.

3.5.4 Hold the talus, dome upwards and medial surface towards the collector, over the weighing tray. Scrape cortical bone from the neck of the talus to a depth of ~ 1mm (see Figure 5) using a dental drill/Dremel with low gauge bit (see Materials Table for example) set to low speed and high torque.

3.5.5 Change drilling position slightly and repeat until approximately 50-100 mg of bone powder as measured using an enclosed balance accurate to the 100th mg (see Materials Table for example) has been collected on weigh paper.

3.5.6 Transfer bone powder from weigh paper to 2ml low-bind tube for extraction. Store at (-20 °C) indefinitely.

3.5.7 Store remaining bone/excess powder in a dry, temperature controlled (25 °C) sterile environment until return/repatriation can be completed.

3.5.8 Dispose of all waste in autoclavable biohazard bags or receptacles. Sterilize/decontaminate all reusable equipment (*e.g.*, clamps, drill bits, drills, saws, *etc.*) using bleach/DNAerase/ethanol and UV (wavelength: ca. 254 nm) exposure, as applicable, between each sampling.

[Insert Figure 5 here].

NOTE: The talus has very little cortical bone (a thin outer layer). As such, not only is surface removal not possible, but material from the underlying dense layer of cancellous bone should also be collected.

Representative Results:

In a separate study[11], DNA was extracted from bone powder generated from each anatomical sampling location in 11 individuals, using a standard DNA extraction protocol optimized for short fragments from calcified tissue[2]. Single-stranded libraries were then produced[28] and sequenced on a HiSeq 4000 (75 bp paired-end) to a depth of ~20,000,000 reads per sample. The resulting sequence data was then evaluated for endogenous human DNA content using the

EAGER pipeline[29] (BWA settings: Seed length of 32, 0.1 mismatch penalty, mapping quality filter of 37). Libraries from the powdered portions of the *pars petrosa* yielded, on average, higher endogenous DNA than any of the other 23 anatomical sampling locations surveyed. The seven additional anatomical sampling locations presented in this protocol (the cementum, first pass of the dental pulp chamber, and dentin of permanent molars; cortical bone from the vertebral body and superior vertebral arch of the thoracic vertebra; cortical bone from the apical tuft of the distal phalanx; and cortical bone from the neck of the talus) produced the next highest yields (with no statistical significance between these anatomical sampling locations). These alternative locations all consistently produced DNA yields adequate for standard population genetics analyses such as mitochondrial analyses and SNP analyses (Figure 6A-B). Duplication rates in libraries stemming from all anatomical sampling locations were low (cluster factors < 1.2 on overage, calculated as the ratio of all mapping reads to unique mapping reads, Table 2, Supplemental File 1: ClusterFactor), indicating that all libraries screened were of very high complexity. Similarly, average exogenous human DNA contamination estimates were low, averaging < 2% (X chromosome contamination in males, n=7, as reported by the ANGSD[30] pipeline) in all but anatomical sampling locations except for the superior vertebral arch (average estimated contamination: 2.11%, with one sample removed as an outlier; KRA005: 19.52%, See Table 2; Supplemental File 1: Xcontamination). Average fragment length (after filtering to remove all reads < 30 bp) was lowest in material collected from the dental pulp chamber and dentin, with no significant variation amongst other anatomical sampling locations (55.14 bp and 60.22 bp, respectively in comparison to an average median of 62.87, pair-wise p-values <0.019, Table 2; Supplemental File 1; AvgFragLength). Additionally, the teeth and thoracic vertebrae

each contain multiple anatomical sampling locations where high endogenous DNA recovery was observed, making them particularly suitable as alternatives to the *pars petrosa*.

[Insert Figure 6 here].

[Insert Table 2 here]

Discussion:

Current practice in ancient human population genetics is to preferentially sample from the *pars petrosa* (Section 2.1) whenever possible. However, the *pars petrosa* can be a difficult sample to obtain, as it is highly valued for a myriad of skeletal assessments (*e.g.*, population history[32], the estimation of foetal age at death[33], and sex determination[34]) and, historically, sampling of the *pars petrosa* for DNA analysis can be highly destructive[3-4] (including the protocol presented here, although new, minimally invasive protocols[13,14] have now been widely adopted to alleviate this concern). This is compounded by the fact that, until very recently, a large-scale, systematic study of human DNA recovery across the skeleton had not been attempted[11], making finding an appropriate sampling strategy when the petrous pyramid is unavailable challenging.

The protocols presented here help to alleviate that challenge by providing a set of optimized procedures for DNA sampling from archaeological/forensic skeletal remains including the *pars petrosa* as well as seven alternate anatomical sampling locations across four additional skeletal elements. The critical steps included are all intended to minimize the possibility of DNA

loss/damage due to either inefficient sampling (Sections 2.1.6 and 3.2.1.3) or overheating of samples during drilling/cutting (Section 3.1.6,). Additionally, it has been noted throughout the protocol that it may be necessary to modify/omit the pre-treatment steps to ensure best performance in highly degraded samples. Additionally, it should be noted that even among the selected elements presented here, there remain several possible alternative sampling techniques (particularly for the *pars petrosa*[13,14]), as well as ample room for further optimization of the underexploited anatomical sampling locations presented here *(i.e.*, the talus: Section 2.5 and the vertebrae: Section 2.3).

It is also important to keep in mind that these protocols have been designed and tested using ancient juvenile-adult remains of high quality (good morphological preservation) for the purposes of endogenous human DNA analyses. The results presented may not extend to more highly degraded materials, other preservation contexts, infant remains, non-human remains, or studies of pathogens or the microbiome, as greater exploration into the use of these protocols in additional contexts is still needed. Additionally, the alternative skeletal elements presented here (the teeth, vertebrae, distal phalanx, and tali) may be challenging to assign to a single individual among commingled remains, necessitating sampling from multiple elements to ensure a single origin. Despite these limitations, making these protocols widely available can help alleviate some of the heterogeneity surrounding sample selection and processing by providing a generalized and quantitatively optimized framework for use in a wide range of future aDNA/forensic studies on human remains.

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Author contributions:

CP developed and optimized all new sampling protocols and is the primary author of the manuscript. WH, KB and JK jointly designed, supervised, and funded this project, as well as aided in editing of the manuscript.

Code Availability:

All analyses programs and R modules used in the analyses of this manuscript are freely available from their respective authors. All custom R code is available by request.

Data Availability:

All raw data used in the calculation of representative results is freely available in the European Nucleotide Archive ENA data repository (accession number PRJ-EB36983) or supplemental materials of:

Parker, C. *et al*. A systematic investigation of human DNA preservation in medieval skeletons. Scientific Reports. 10 (1), 18225, doi: 10.1038/s41598-020-75163-w (2020).

Statement of Conflicts of Interests:

The authors have no conflicts of interest to report

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Figure 1A-B. Temporal bone including the *pars petrosa*. (A) Sample pre-cutting showing the locations of the petrous pyramid and the *sulcus petrosa* (B) Petrous portion post-cutting highlighting the dense areas to be drilled.

Figure 2A-C. Permanent molar pre-sampling. (A) Pre-treated molar prior to sampling, showing crown, cementum (yellowish layer of root), and the cutting site at the cemento-enamel junction. (B) The same molar post-cementum collection, showing the cut site at the cemento-enamel junction. (C) Molar post-cutting and sampling showing anatomical sampling locations for the dental pulp chamber and dentin within the crown.

Figure 3. Vertebral body and superior vertebral arch cortical bone anatomical sampling locations of the thoracic vertebra.

Figure 4. Distal phalanx showing the locations of dense cortical bone along the shaft and inferior side of the apical tuft.

Figure 5. Sampling area of the talus for cortical bone recovery.

Figure 6A-B. Human DNA content for all screened samples. Black lines represent the overall mean, red the median (solid: human DNA proportion, dashed: mapped human reads per million reads generated). Individual anatomical sampling locations with an average human DNA proportion higher than the overall mean (8.16%) are colorized in all analyses. A) Proportion of reads mapping to the hg19 reference genome. The blue dashed line represents the theoretical maximum given the pipeline's mapping parameters (generated using Gargammel[31] to simulate a random distribution of 5,000,000 reads from the hg19 reference genome with simulated damage). Individual means (black X) and medians (red circle) are reported for those samples with a higher average human DNA proportion than the overall mean. Confidence intervals indicate upper and lower bounds excluding statistical outliers. B) Number of unique reads mapping to the hg19 reference genome per million reads of sequencing effort (75bp paired end Illumina). Confidence intervals indicate upper and lower bounds excluding statistical outliers. Figure adapted from Figure 2 in Parker, C. et al. A systematic investigation of human DNA preservation in medieval skeletons. Scientific Reports. 10 (1), 18225, doi: 10.1038/s41598-020-75163-w (2020).[11]

Table 1. Genetically determined sex archaeologically determined estimated age at death, and radiocarbon dating (14C Cal 2-sigma) for all eleven individuals sampled from. Adapted from Table 1, Parker, C. et al. A systematic investigation of human DNA preservation in medieval skeletons. Scientific Reports. 10 (1), 18225, doi: 10.1038/s41598-020-75163-w (2020).[11]

Table 2. Average duplication levels (mapping reads/unique reads), average and median fragment lengths, and X chromosome contamination estimates for all anatomical sampling locations. Error reported as standard error of the mean. Adapted from Table 2, Parker, C. et al. A systematic investigation of human DNA preservation in medieval skeletons. Scientific Reports. 10 (1), 18225, doi: 10.1038/s41598-020-75163-w (2020).[11]

Sheet1

Sample	Sex	Source	mgInput	RawReads	Duplicates	Mapped
KRA001.D05(	M	Superior Vert	43.2	45015292	975092	5840195
KRA003.D05(	M	Superior Vert	59.9	47661406	977426	5657343
KRA004.D05(	M	Superior Vert	47.8	41254478	87384	391440
KRA005.D05(	M	Superior Vert	54.6	46095622	227287	834432
KRA008.D05(	M	Superior Vert	61.6	56577278	614117	1809455
KRA009.D05(	M	Superior Vert	51.7	41642702	478062	1880302
KRA010.D05(	M	Superior Vert	48.3	48754502	1587736	9060486
KRA002.D05(	F	Superior Vert	60	41708522	199321	1202118
KRA006.D05(	F	Superior Vert	52.9	43536234	58730	347438
KRA007.D05(	F	Superior Vert	67.1	40995082	107895	594496
KRA011.D05(	F	Superior Vert	56.6	41254284	63160	366976
KRA001.A01(	M	Cementum	26.1	35093372	1011283	5306782
KRA003.A01(	M	Cementum	17.8	49348472	1846518	4822790
KRA004.A01(	M	Cementum	44.2	48792028	1906481	6908709
KRA005.A01(	M	Cementum	16	38724638	2642633	8497581
KRA008.A01(	M	Cementum	8.1	43610850	2632935	8615057
KRA009.A01(	M	Cementum	10.8	33924664	514632	1888015
KRA010.A01(	M	Cementum	12.3	25763474	1425477	5742316
KRA002.A01(	F	Cementum	18.6	46796722	854030	3765592
KRA006.A01(	F	Cementum	28	50656116	632837	2376535
KRA007.A01(	F	Cementum	7.5	47139534	1765667	3498398
KRA011.A01(	F	Cementum	24.3	38294228	47272	266095
KRA001.A02(	M	Dentin	52.9	42734006	909075	5739310
KRA003.A02(	M	Dentin	50	46041498	33336	172855
KRA004.A02(	M	Dentin	44.6	46966864	6310	34550
KRA005.A02(	M	Dentin	43.6	48893384	1700187	2519823
KRA008.A02(	M	Dentin	60.3	37186642	1339396	8306879
KRA009.A02(	M	Dentin	43.5	40200238	1020478	3319127
KRA010.A02(	M	Dentin	81.4	44918134	1314184	7371721
KRA002.A02(	F	Dentin	42.5	46898212	515899	2708055
KRA006.A02(	F	Dentin	46.8	44042542	71881	344855
KRA007.A02(	F	Dentin	41.4	45221170	1169950	1701203
KRA011.A02(	F	Dentin	76.4	44421388	781029	4640099
KRA001.G01(	M	Distal Phalan	27.7	36717038	227653	1373072
KRA003.G01(	M	Distal Phalan	69.6	50109386	1272496	1732567
KRA004.G01(	M	Distal Phalan	26.2	40459678	1437182	7526954
KRA005.G01(	M	Distal Phalan	41.6	38973980	1580443	8736523
KRA008.G01(	M	Distal Phalan	53	42606726	1624009	7721887
KRA009.G01(	M	Distal Phalan	43	42871168	2243429	10049025
KRA010.G01(	M	Distal Phalan	56.1	37195858	1074040	6022589
KRA002.G01(	F	Distal Phalan	49.9	42969640	635742	2848396
KRA006.G01(	F	Distal Phalan	70	42305802	898514	4961871
KRA007.G01(	F	Distal Phalan	45.7	41470730	874852	3256494
KRA011.G01(	F	Distal Phalan	35.3	41215792	830538	3051705
KRA001.B01(	M	Petrous	50	38633714	1211833	8221604
KRA003.B01(	M	Petrous	48.3	45338996	177801	981008
KRA004.B01(	M	Petrous	63	41880152	1395790	7792266
KRA005.B01(	M	Petrous	63	39844482	1419640	9780339
KRA008.B01(	M	Petrous	47.1	38677032	401173	3130535
KRA009.B01(	M	Petrous	48.7	46905290	282483	1458343
KRA010.B01(	M	Petrous	60.1	47849002	1172592	7811241
KRA002.B01(	F	Petrous	51.1	45480366	513475	2969672
KRA006.B01(	F	Petrous	54.6	42381100	399466	2740240
KRA007.B01(	F	Petrous	52.3	38805502	923106	6206382

KRA011.B01(	F	Petrous	58.1	38286878	1220093	7655339
KRA001.A03(	M	Pulp	15.7	41062240	628965	4717206
KRA003.A03(	M	Pulp	11.4	48534722	65064	347166
KRA004.A03(	M	Pulp	45.8	45617030	194441	1326259
KRA005.A03(	M	Pulp	7.8	53522980	1049034	2492194
KRA008.A03(	M	Pulp	38.9	44817806	782979	3030359
KRA009.A03(	M	Pulp	53	38041402	702061	4493823
KRA010.A03(	M	Pulp	20.9	47733170	1541158	9098301
KRA002.A03(	F	Pulp	20.8	41559480	281262	1606790
KRA006.A03(	F	Pulp	14	42860294	19032	84943
KRA007.A03(	F	Pulp	15.7	43417840	4864	26734
KRA011.A03(	F	Pulp	17.6	50213906	1625803	7450640
KRA001.J010	M	Talus	77.9	43227720	398221	1990249
KRA003.J010	M	Talus	45.9	44532528	695817	3782670
KRA004.J010	M	Talus	49	34685948	1098309	5902769
KRA005.J010	M	Talus	58.7	49111232	2191830	12007405
KRA008.J010	M	Talus	48	44713028	476293	1672613
KRA009.J010	M	Talus	68.4	41216448	2170807	10816421
KRA010.J010	M	Talus	57.3	41892026	1130201	6202971
KRA002.J010	F	Talus	74.5	43330172	222625	1125931
KRA007.J010	F	Talus	58.8	36318224	906355	4581352
KRA011.J010	F	Talus	71.3	52976394	1427182	5927603
KRA001.D02(	M	Vertebral Bod	43.3	45987428	633101	3452695
KRA003.D02(	M	Vertebral Bod	48	42841202	1655692	8611934
KRA004.D02(	M	Vertebral Bod	59.6	49291326	1672105	3293124
KRA005.D02(	M	Vertebral Bod	39.3	45382348	1579419	7414195
KRA008.D02(	M	Vertebral Bod	51	38533108	1305312	6514498
KRA009.D02(	M	Vertebral Bod	53.2	37514150	1049854	5073584
KRA010.D02(	M	Vertebral Bod	63.2	39803670	1252698	6055528
KRA002.D02(	F	Vertebral Bod	44.3	40797230	430248	2266995
KRA006.D02(	F	Vertebral Bod	41	41978776	1138702	6305851
KRA007.D02(	F	Vertebral Bod	44.9	45738280	794154	4816825
KRA011.D02(	F	Vertebral Bod	69	47540272	438830	924516

EndogenousD	EndogenousD	OnTarget	ClusterFactor	MeanCoverag	MT/NUCRatic	Xcontaminatic
23.842	47.424	16.392	1.167	1.6933	66.88	0.024136
15.323	40.261	14.207	1.173	1.6643	170.19	0.005453
0.642	3.494	1.356	1.223	0.1272	175.55	0.065458
0.66	6.101	2.536	1.272	0.2909	126.13	0.19524
1.894	14.457	5.512	1.339	0.5747	216.97	0.012859
2.595	18.312	7.071	1.254	0.6176	51.82	0.008328
43.461	55.213	15.507	1.175	2.1374	94.99	0.010644
2.018	8.409	3.052	1.166	0.3682	106.22	0.285068
1.909	2.375	0.759	1.169	0.0938	100.65	0.283424
1.688	6.169	2.114	1.181	0.1714	166.16	0.230371
1.984	3.221	0.836	1.172	0.0806	147.77	0.226249
19.319	45.945	14.084	1.191	1.3754	144.48	0.005551
7.155	39.015	13.703	1.383	1.3143	608.65	0.01272
13.802	42.13	12.86	1.276	1.7626	186.18	0.00215
64.403	73.123	22.238	1.311	1.9684	217.36	0.005969
54.933	70.017	20.804	1.306	1.9599	191.13	0.010717
2.789	18.391	6.631	1.273	0.5764	77.89	0.023571
49.262	68.966	22.837	1.248	1.5479	128.61	0.013534
7.71	30.189	11.768	1.227	1.1721	110.66	0.259303
16.441	16.066	5.239	1.266	0.6416	202.46	0.248849
5.434	33.854	12.026	1.505	0.9033	721.39	0.253597
1.015	1.872	0.533	1.178	0.0645	132.62	0.203662
19.32	39.398	11.544	1.158	1.4037	610.95	0.004713
0.63	1.37	0.39	1.193	0.0411	12025.7	-0.039481
0.078	0.21	0.072	1.183	0.0099	7367.73	0
2.645	21.606	7.947	1.675	0.7609	8949.55	0.013321
47.761	57.581	16.432	1.161	2.0008	524.58	0.012126
4.703	27.955	11.406	1.307	1.1038	3996.34	0.021558
32.368	50.084	13.104	1.178	1.5886	1491.29	0.005395
6.654	17.982	6.163	1.191	0.7604	581.42	0.287608
2.84	2.299	0.827	1.208	0.1045	2602.83	0.256517
2.303	17.558	6.064	1.688	0.4398	19089.77	0.212883
18.854	31.949	10.363	1.168	1.2588	205.54	0.258701
2.898	11.028	3.657	1.166	0.3873	53.58	0.039452
1.97	20.104	8.228	1.734	0.4828	222.88	0.015628
28.842	55.807	19.486	1.191	2.2177	64.95	0.002768
32.911	61.858	22.271	1.181	2.6587	86.12	0.000646
29.307	54.043	18.255	1.21	2.2131	78.57	0.018937
56.111	66.764	17.98	1.223	2.2903	31.64	0.005541
19.169	49.497	18.755	1.178	1.9171	52.57	0.0053
5.705	22.006	7.092	1.223	0.7793	85.38	0.271575
15.834	36.69	11.668	1.181	1.3393	78	0.260428
6.761	27.686	10.049	1.269	0.9882	67.35	0.242231
5.01	25.479	9.858	1.272	0.9715	79.39	0.264606
57.15	60.855	14.093	1.147	1.6096	110.45	0.009679
3.539	7.536	2.539	1.181	0.2811	69.06	-0.012294
41.275	52.281	14.342	1.179	1.8098	94.36	0.006868
62.932	65.784	16.56	1.145	2.0861	106.86	0.00791
25.006	18.254	2.564	1.128	0.3701	128.94	-0.01221
9.538	7.329	1.018	1.194	0.1704	78.74	-0.004622
44.453	52.42	12.767	1.15	1.5919	113	-0.000507
9.221	19.472	5.778	1.173	0.7415	95.74	0.277828
16.375	22.655	5.574	1.146	0.5667	109.38	0.251434
41.215	47.892	12.85	1.149	1.4048	149.86	0.274287

57.954	47.04	10.148	1.159	1.3967	66.6	0.27868
25.366	35.77	8.884	1.133	0.9803	324.66	0.015878
1.879	2.811	0.953	1.187	0.0974	6076.92	0.042001
3.268	8.654	2.549	1.147	0.3262	141.42	-0.000246
2.846	19.827	7.893	1.421	0.7914	5144.58	0.005486
4.82	20.447	7.256	1.258	0.9112	597	-0.000778
17.604	36.935	10.97	1.156	1.1186	117.03	0.008975
43.236	52.929	13.263	1.169	1.9273	134.33	0.016928
3.948	12.406	4.067	1.175	0.4302	1151.12	0.278288
0.598	0.635	0.25	1.224	0.0281	2919.47	0.27687
0.3	0.27	0.075	1.182	0.0063	9904.9	0
39.607	50.237	17.078	1.218	2.1134	67.06	0.293787
2.645	16.644	5.556	1.2	0.5646	65.07	0.008067
18.562	23.054	6.889	1.184	0.9591	164.13	0.021629
17.296	47.275	16.073	1.186	1.6981	71.47	0.003335
66.148	71.013	18.992	1.183	2.7064	111.63	0.005543
2.465	14.865	6.127	1.285	0.5711	243.93	0.011208
61.362	69.261	17.78	1.201	2.3474	78.35	0.003686
17.04	43.69	15.073	1.182	1.8042	127.31	0.021729
1.426	7.646	2.694	1.198	0.3348	394.4	0.290863
24.503	49.365	15.478	1.198	1.2185	74.49	0.264885
13.628	36.312	11.734	1.241	1.5971	100.41	0.262463
11.04	27.249	9.008	1.183	0.9692	76.94	0.016327
40.084	62.12	22.777	1.192	2.6643	56.68	0.003889
4.507	27.56	10.751	1.508	0.9274	58.32	0.001929
21.242	52.954	19.012	1.213	2.2132	40.81	0.002002
32.222	56.096	19.958	1.2	1.9572	58.25	0.006668
16.124	45.392	15.912	1.207	1.5107	63.59	0.019183
20.253	45.897	15.572	1.207	1.7392	81.95	0.009274
3.031	16.04	5.739	1.19	0.6881	117.69	0.252238
21.065	49.106	17.903	1.181	1.9333	69.65	0.260835
28.536	37.058	8.077	1.165	0.8853	54.4	0.246653
0.92	8.244	3.339	1.475	0.3029	137.89	0.312926

AvgFragLength
62.66
66.49
59.54
59.57
64.9
63.25
59.79
60.66
73.32
62.42
60.66
73.18
57.12
73.54
76.36
65.02
69.99
61.19
67.41
69.02
62.87
64.37
57.75
52.98
47.04
51.13
69.01
66.01
70.04
69.24
62.77
57.52
58.93
69.56
67.97
59.87
66.37
66.39
68.56
69.15
66.96
68.53
61.74
60.35
68.43
66.31
67.72
66.13
66.98
61.37
64.31
64.03
68.33
54.36

71.39
54.88
49.35
40.42
50.53
47.51
63.94
49.01
74.72
56.66
64.96
54.61
75.14
65.29
72.46
66.73
65.7
65.15
62.9
72.25
68.92
67.41
70.01
72.12
65.83
60.57
64.44
67.14
66.44
63.93
68.39
66.91
61.72

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Thank you for your consideration of our manuscript,

Cody Parker, Dr. Kirsten I Bos, Dr. Wolfgang Haak, Prof. Johannes Krause

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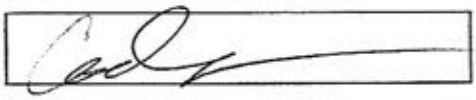
Signature:	
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