MOLECULAR ECOLOGY
RESOURCES

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Journal:	*Molecular Ecology Resources*
Manuscript ID	MER-16-0205.R1
Manuscript Type:	Resource Article
Date Submitted by the Author:	n/a
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Combining bleach and mild pre-digestion improves ancient DNA recovery from bones

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Running title: Improved access to ancient DNA

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Abstract

The feasibility of genome-scale studies from archaeological material remains critically dependent on the ability to access endogenous, authentic DNA. In the majority of cases, this represents a few percent of the DNA extract, at most. A number of specific pre-extraction protocols for bone powder aimed to improve ancient DNA recovery before library amplification have recently been developed. Here, we test the effects of combining two of such protocols, a bleach wash and a pre-digestion step, on 12 bone samples of Atlantic cod and domestic horse aged 750-1350 cal. years before present. Using high-throughput sequencing, we show that combined together, bleach wash and pre-digestion consistently yield DNA libraries with higher endogenous content than either of these methods alone. Additionally, the molecular complexity of these libraries is improved and endogenous DNA templates show larger size distributions. Other library characteristics, such as DNA damage profiles or the composition of microbial communities are little affected by the pre-extraction protocols. Application of the combined protocol presented in this study will facilitate the genetic analysis of an increasing number of ancient remains and will reduce the cost of whole genome sequencing.

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Introduction

Archaeological bone material represents the primary source of ancient DNA (aDNA) (e.g. Hofreiter, et al. 2014; Der Sarkissian, et al. 2015). High-throughput DNA sequencing of aDNA extracts typically allows for the recovery of portions of the nuclear and mitochondrial genomes. The practical and financial feasibility of such studies is critically dependent on our ability to retrieve the degraded DNA molecules that survived after death. While petrosal bones represent exceptions (e.g. Reich, et al. 2010; Meyer, et al. 2012; Gamba, et al. 2014; Lazaridis, et al. 2014; Pinhasi, et al. 2015), the fraction of endogenous DNA present in bones, also referred to as their endogenous content, is typically low and in the range of a few percent at most (Carpenter, et al. 2013). Consequently, a major challenge of working with ancient bone material is maximizing cost-efficiency when using high-throughput sequencing at genome-wide scales (Rizzi, et al. 2012).

In recent years, continuing attention has been paid to developing novel experimental methods to improve access to the endogenous content from ancient samples. For instance, hybridization capture techniques aim to enrich entire genomes or specific sections after library preparation by annealing templates to pre-selected DNA baits (Briggs, et al. 2009; Carpenter, et al. 2013; Enk, et al. 2014). Other approaches improve access to the endogenous DNA fraction at the extraction stage, which minimizes ascertainment bias and other experimental bias associated with capture based methods (Castellano, et al. 2014). As such, the surface of bone samples is typically cleaned by mechanical removal of the outer layer, and the application of chemical and/or enzymatic treatments (Kemp and Smith 2005; Malmström, et al. 2007; Li and Liriano 2011). More recently, methods to clean bone powder – i.e. after homogenization of the bone sample – have been shown to significantly increase the fraction of endogenous templates sequenced, especially through the washing of bone powder with

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bleach or phosphate (Korlevic, et al. 2015), or a proteinase K "pre-digestion" step prior to DNA extraction (Orlando, et al. 2011; Ginolhac, et al. 2012; Der Sarkissian, et al. 2014; Gamba, et al. 2014; Damgaard, et al. 2015; Gamba, et al. 2016). The success of these methods indicates that endogenous DNA is more tightly bound to the bone matrix than exogenous DNA, perhaps in micro-niches in the bone material (Campos, et al. 2012; Ginolhac, et al. 2012) and provides a rationale to further investigate simple, cost-effective washing methods for the pre-treatment of ancient bone material.

Here, we apply high-throughput sequencing to investigate the efficiency of a combined bleach wash and pre-digestion on the endogenous content of 12 ancient bone samples from Atlantic cod (*Gadus morhua*) and horse (*Equus caballus*), dated to 750-1350 cal. years before present (BP) and coming from various archaeological contexts. We show that combined together, bleach wash and pre-digestion consistently increase the endogenous DNA content. This procedure leads to a 1.7 to 3-fold increase relative to the pre-digestion protocol in absence of a bleach wash. This improvement is not associated with a significant increase in DNA damage patterns or changes in the composition of microbial communities. Our results indicate that a multiplicative effect enriches endogenous DNA prior to library build, and highlight the effectiveness of treating bone powder prior to DNA extraction.

Material and methods

Sample information

Bones from Atlantic cod (*Gadus morhua*) and horse (*Equus caballus*) were obtained from Quoygrew and the Brough of Deerness, Orkney, Scotland, and the Gokstad ship burial, Norway (Table S1, Supporting Information; Bonde and Christensen 1993; Barrett and Slater 2009; Barrett 2012). Age estimates (between 1350 and 750 cal. years BP) were indirectly

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obtained based on associated archaeological remains. We ensured that bones from independent individuals were collected, by using the same bone type within a stratum or bones from different strata within the archaeological site.

Sampling and DNA extraction

All laboratory work preceding PCR was done in the dedicated ancient DNA laboratories at the Institute of Biosciences, University of Oslo following strict DNA precautions (Poinar and Cooper 2000; Gilbert, et al. 2005). Bone samples were exposed to UV for 10 minutes on each side resulting in a total dosage of 4800 J/m2 before being cut and milled to powder. Atlantic cod bones were milled completely in a Retsch MM400, while horse bones or teeth were subsampled (cutting out a part of the root) using a scalpel attached to a Perfecta 600 (W&H). The surface of each subsample was removed using a drill bit. All subsamples were subsequently exposed to UV (describe above) before being homogenised in a Retsch MM400 mixer mill. Washes and DNA extractions were performed on 200 mg of homogenized bone powder unless stated otherwise.

DNA extraction experiments

DNA was extracted from powder from five Atlantic cod samples and seven horse samples after two different treatments: 1) a double digestion, whereby the bone powder remaining after a first, short digestion or pre-digestion step was subjected to a second digestion using a fresh digestion buffer (DD), and 2) a bleach wash followed by a double digestion (BleDD); Fig. 1). For the cod samples, we also extracted DNA from the supernatant of the first, short digestion, both when bone powder was subjected only to digestion directly after homogenization (short digestion, SD) and when bone powder was washed with bleach prior

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to the short digestion step (BleSD). Finally, we tested the effect of adding twice the amount (i.e. two times 200 mg) of starting material for Atlantic cod bones when using BleDD (BleDD^2; Fig. 1).

Washing of bone powder with bleach was carried out by incubating 150-200 mg bone powder in 1 ml of 0.5% bleach solution (VWR) in 2 ml tubes for 15 min at room temperature (RT) under gentle 3-dimensional shaking using a nutating mixer. After incubation, bleach was removed by centrifugation and removal of supernatant and washing of bone powder repetitively (three times) with 1 ml UV-treated milli-Q water, whereby thorough contact between water and bone powder was ensured by vortexing before centrifugation, as per Korlevic et al. (2015). For BleDD^2, bleach wash and digestion with 200 mg of bone powder were performed in two separate tubes and combined following the second digestion (see below).

The short, pre-digestion treatment of samples following Damgaard, et al. (2015) was carried out in 1 ml volumes containing 0.45 M EDTA (pH 8.0), 25 µl proteinase K (~18 mg/ml; Roche) and 0.5% N-Laurylsarcosyl. Incubations were performed at 37°C under gentle 3-dimensional shaking for one hour. Following centrifugation, the supernatant was directly transferred to a new tube for extraction of DNA (SD and BleSD protocols) or stored at -20°C for later extraction. Following the removal of the supernatant from this first, short digestion, the remaining bone powder was subjected to an overnight, second digestion using a freshly prepared digestion buffer (DD, BleDD and BleDD^2 protocols). Following Gamba, et al. (2016), in all protocols the supernatant was concentrated to 200 µl by adding it to 2 ml 10 mM Tris-EDTA (Sigma-Aldrich) and spinning through an Amicon Ultra 4 (30 kD). DNA was subsequently extracted using Qiagen Minelute columns according to manufacturer's

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instructions, and eluted in 60 ųl pre-heated (60°C) EB buffer after a 10 minute incubation at 37°C. Negative controls were included in all extraction experiments.

Library preparation and sequencing

Blunt-end Illumina libraries as described by Meyer and Kircher (2010) were built following Schroeder, et al. (2015) with the exception that all reactions were performed in half volumes. Sample-specific seven bp indexes in the P7 primer were used to allow multiplexing of libraries for sequencing. Library amplification was performed in 15 µl of ligated DNA with 2.5 U PfuTurbo Cx Hotstart DNA Polymerase (Agilent Technologies), 1x buffer, 0.2 mM per dNTP, 0.2 µM P7 index primer, 0.2 µM P5 IS4 primer and 0.4 mg/ml BSA. Reactions were amplified for 13 cycles (2 min at 95°C, 13 cycles of 30s at 95°C, 30s at 60°C and 70s at 72°C with a final extension of 10 min at 72°C). Amplified library products were subsequently cleaned using Agencourt® AMPure XP beads at a 1:1.7 ratio (PCR volume: AMPure volume), eluted in 30µl sterile Millipore water and visualised and quantified on a Bioanalyzer 2100 (Agilent). Subsets of libraries that required further amplification due to low concentration were split over four separate reactions to minimize library clonality. These re-amplifications were performed using 1 U Accuprime Pfx DNA polymerase (Invitrogen), 1x buffer, 0.25 µM IS5, 0.25 µM IS6 and 1 unit polymerase) and amplified with the following PCR profile: 2 min at 95°C, 4 cycles of 20s at 95°C, 20s at 60°C and 40s at 72°C, final extension step of 3 min at 72°C (see Tables 1 and 2 for the total number of cycles each library was amplified for). Re-amplified libraries were pooled before cleanup with AMPure as described above. Sequencing of libraries was carried out on an Illumina Hiseq 2500 at the Norwegian Sequencing Centre (125 bp paired-end) and data were demultiplexed following standard procedure allowing no mismatches in the index sequences. To monitor

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161 contamination libraries were also built of DNA extraction negative controls and these were
162 sequenced along with the sample libraries. Sequence reads from these controls were analysed
163 as reads from sample libraries (see below) and yielded less than 0.0094 % and 0.016 % of
164 reads mapping back to the genomes of horse and cod, respectively.
165
166 *Analyses*
167 Sequence reads were processed using the PALEOMIX pipeline v. 1.2.4 (Schubert, et al.
168 2014). Adapters were removed and reads were collapsed and trimmed as described in
169 (Orlando, et al. 2013) using AdapterRemoval version 2.1.7, discarding collapsed reads below
170 25 bp in length (Schubert, et al. 2016). Collapsed reads were down sampled to the lowest
171 number of reads (894,682) and mapped to EquCab2.0 (Wade, et al. 2009) and GadMor2
172 (Tørresen, et al. 2016) excluding the mitochondrial genome, using the backtrack algorithm
173 from BWA v.0.5.10 (Li and Durbin 2009) with seeding disabled as implemented in
174 PALEOMIX. Sequence reads were considered endogenous if they aligned to their respective
175 reference genome with a minimum alignment quality (MinQ value) of 25. Duplicates were
176 removed and a variety of summary statistics including endogenous DNA content, clonality,
177 average read length and GC-content was directly provided by PALEOMIX. Statistical tests
178 were applied when comparing treatments (paired t-test, or Wilcoxon Signed Rank if normality
179 is rejected using the Shapiro-Wilk normality test (Shapiro and Wilk 1965)). Critical
180 significance levels were adjusted using Bonferroni's correction for the number of multiple
181 tests in this study.
182 We estimated library complexity in sequence data obtained from three treatments
183 (DD, BleDD, BleDD^2). Library complexity predictions from small datasets of shallow
184 sequencing can give false estimates of library complexity (Daley and Smith 2013). We

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185 therefore down sampled the collapsed sequencing files of each sample-treatment combination
186 (using seqtk, https://github.com/lh3/seqtk), matching the lowest number of sequences *per*
187 *individual sample*, instead of matching the lowest number in the entire dataset (as above), to
188 allow for a direct comparison of sequencing efficiency (defined as the fraction of non-clonal
189 endogenous DNA sequences divided by total sequences) using as much data as possible.
190 Several signatures of DNA damage were explored to investigate if any of the
191 treatments result in compositional bias or introduced further damage to the DNA. We
192 obtained read-length distributions, aDNA damage patterns and calculated the probability of
193 observing cytosine deamination in double strand context (δd), cytosine deamination in single
194 strand context (δs), and the probability of terminating in a single-stranded overhang (λ) using
195 mapDamage v.2.0.6 (Ginolhac, et al. 2011; Jonsson, et al. 2013). Atlantic cod and horse
196 alignment files were down sampled to 1,000,000 and 500,000 reads, respectively, when more
197 reads were sequenced.

198 *Microbial analyses*

199 We investigated the effect of the different pre-extraction treatments on the microbial
200 composition in the read data obtained from Atlantic cod and horses. We calculated relative
201 abundances by running the microbial profiling pipeline metaBIT (Louvel, et al. 2016),
202 aligning collapsed reads using Bowtie2 v2.1.0 (Langmead and Salzberg 2012) to the
203 MetaPhlAn2 database (version 2.0, excluding eukaryotes and viruses Truong, et al. 2015) and
204 excluding PCR duplicates with a modified version of the FilterUniqueBAM Python script
205 from PALEOMIX (Schubert, et al. 2014). Microbial profiles were generated at all
206 taxonomical levels from kingdom to strain using MetaPhlAn (Segata, et al. 2012; Truong, et
207 al. 2015), excluding low-abundance taxa (<1%) to reduce statistical noise due to false
208 positives. These profiles were compared to 689 profiles from the Human Microbiome Project

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(Consortium 2012) and 15 profiles from soil samples (Fierer, et al. 2012; Louvel, et al. 2016) to detect possible human microbial contamination from handling of the specimens and the depositional context using Principal Coordinate Analyses (PCoA) of Bray-Curtis distances with the R function *pcoa*. We also compared microbial profiles by hierarchical clustering with an average linkage clustering method and 10,000 bootstrap iterations using the R package *pvclust* (Suzuki and Shimodaira 2006) adapted for Bray-Curtis distances in metaBIT (Louvel, et al. 2016).

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Results

For each DNA extraction –following a short pre-digestion (SD), a double digestion (DD), bleach wash followed by a short pre-digestion (BleSD) or a double digestion (BleDD), or BleDD with twice the amount of starting material (BleDD^2)– between 1 and 36 million collapsed reads were obtained (Table S2, Supporting Information). Washing bone powder with bleach (comparing DD v BleDD) significantly (paired t = -4.095, DF = 11, p = 0.0026, Fig. 2) increased the proportion of high quality and uniquely mapped endogenous reads, after correcting for multiple significance tests (n = 15, corrected p-value = 0.0033). BleDD treatment increased the proportion of endogenous DNA by 1.3 to 3-fold relative to DD in all but one of the samples (sample COD034, Fig. 3). No significant difference in clonality (Wilcoxon Signed Rank, W = 29, N = 12, $p > 0.05$; Fig. 2) or %GC was observed between DD and BleDD (paired t = 2.172, DF = 11, p = 0.054; Fig. S1, Supporting Information). Unsurprisingly, libraries created after doubling the amount of starting material (BleDD^2) needed fewer amplification cycles, consistent with the increased amount of templates available. These libraries showed reduced clonality levels and a further increase in the proportion of high-quality and uniquely mapping endogenous DNA. Library efficiency shows an average 1.8 (BleDD) to 2.5 (BleDD^2) fold enrichment compared to DD (Fig. 3), when measured as the fraction of high-quality and uniquely mapping reads obtained after down sampling to match the lowest number of collapsed reads per bone.

The Atlantic cod libraries created after a short digestion (SD and BleSD) had substantially lower endogenous content relative to their respective double digest treatments (DD, BleDD or BleDD^2, Fig. 2). These libraries also differed in several other characteristics. The mean %GC of both short digestion treatments (SD and BleSD) was significantly higher (paired t = 6.449, DF = 4, p = 0.0031, Fig. S3, Supporting Information) compared to the

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241 double digestion treatments (DD, BleDD and BleDD^2). Furthermore, the mean length of the
242 endogenous DNA reads was significantly lower in the short digestion treatments (SD and
243 BleSD) than those from DD, BleDD and BleDD^2 (paired t = -16.56, DF = 4, p = 7.78E-5, Fig.
244 4). It is noteworthy that the distinct ~10 bp periodic peaks in length that are associated with
245 nucleosome protection (Pedersen, et al. 2014) were not present in both SD and BleSD
246 libraries in most cod samples (with the exception of the BleSD treatment in COD027), but
247 were present in all libraries after double digestion (with or without bleach). Such periodic
248 profiles were not found in all horse samples (Fig. 4), likely reflecting different DNA
249 preservation conditions. Mean length of endogenous DNA was not significantly different
250 between DD and BleDD for the Atlantic cod and horse samples combined (paired t = -2.36,
251 DF = 11, p = 0.039, which is not significant after Bonferroni correction).

252 All aDNA libraries showed the typical aDNA fragmentation and mis-incorporation
253 patterns, regardless of treatment (see COD029 as a representative example in Fig. S2,
254 Supporting Information). The mean probability of cytosine deamination in *double* strand
255 context (δd), cytosine deamination in *single* strand context (δs), and the mean probability of
256 terminating in an overhang (λ) did not differ significantly after bleach treatment in the
257 Atlantic cod and horse samples (Table S3, Supporting Information) and bleach did not
258 consistently lead to damage probabilities for δd, δs and λ outside of the range observed in
259 non-bleach treatments (see also Fig. S3 and Fig. S4, Supporting Information for the 95%
260 confidence intervals for simulated posterior distributions of δd, δs and λ per sample and
261 treatment).

262

263 *Microbial analyses*

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The mean abundance of reads that are classified as microbial using metaBIT did not differ significantly between DD and BleDD (Wilcoxon Signed Rank, $W = 19$, $N = 12$, $p > 0.05$) or between short digestion (SD and BleSD) or double digestion treatments (DD, BleDD and BleDD^2; paired t = 0.93, DF = 4, $p = 1$, Fig. 4). In contrast, the mean abundance of microbial reads was significantly higher for Atlantic cod compared to horse (t = -9.05, DF = 10, p = 9.28E-05, Fig. 5). The microbial profiles generated from the ancient cod and horse samples fall outside the diversity of human microbiomes and cluster closely to those obtained from soil (Fig. S5, Supporting Information). Nonetheless, we obtained no support for segregation of microbial profiles by sample, species (cod or horse), or pre-extraction treatment type following hierarchical clustering, with all bootstrap support below 63% (Fig. S6, Supporting Information). The most abundant microbial species belonged to the genera *Strepsporangium*, *Pseudomonas*, *Brevibacterium* and *Sinobacterium* (Fig. S7, Supporting Information). Profiles from Atlantic cod contained several less abundant genera (e.g., *Marinobacter*, *Halomonas* and *Idiomarina*) that have been associated with the marine environment (Fig. S7, Supporting Information).

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Discussion

In this study, we found that washing ancient bone powder with a combined treatment of bleach and short pre-digestion provides a simple, cost-effective approach that significantly improves the accessible endogenous DNA content of ancient samples. The obvious risk of such treatments lies in the potential loss of endogenous DNA during these washing steps, which we tracked here by sequencing the removed supernatant (SD and BleSD). Our analyses showed that the gains realized by removing a larger proportion of exogenous contaminants relative to endogenous DNA far exceeds the loss of endogenous DNA. Indeed, we observed that the proportion of accessible unique reads after bleach treatment increased in all samples, signifying that the short bleach treatment did not compromise complexity among the template molecules. In particular for the BleDD^2 treatment of the Atlantic cod samples –whereby we used twice the amount of input material with an aim to augment template availability and, thus, the library complexity– gains in efficiency were on average 2.5 fold higher relative to the DD treatment. These improvements appear to be multiplicative to the earlier recorded improvements arising from the use of a pre-digestion approach alone (Der Sarkissian, et al. 2014; Gamba, et al. 2014; Damgaard, et al. 2015; Gamba, et al. 2016).

There have been conflicting observations regarding the potential of bleach to cause DNA damage during treatment. While it has been suggested that bleach could introduce DNA damage similar to those accumulated in aDNA templates (Garcia-Garcera, et al. 2011), this effect has not been replicated in other studies (e.g. Kemp and Smith 2005; Korlevic, et al. 2015). Here too, we obtained no evidence for increased or altered fragmentation processes; no consistent increases in cytosine deamination rates following bleach washing; and no altered %GC after bleach treatment. Overall, sequence bias due to bleach treatment was negligible,

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and sequence data obtained after such treatment does not require specific modifications in post-sequencing analyses.

We observed that the length distribution of endogenous DNA reads obtained for Atlantic cod after the short digestion treatment (SD and BleSD) was significantly shorter, and contained higher %GC than those obtained after the double digest (DD, BleDD, BleDD^2), with little effect of bleach. Moreover, we found that the distinct patterns of a ~10 bp read-length periodicity –which are associated with nucleosome protection (Pedersen, et al. 2014)– were more apparent after double digestion. Our results show that DD treatment is associated with longer, more complex aDNA templates that in Atlantic cod are also more profoundly associated with nucleosome protection. Interestingly, our results indicate that treating samples with a double digest could *increase* rather than *decrease* the potential to obtain epigenetic information from aDNA sequence data, and that bleach does not hinder such applications.

It is hypothesized that (microbial) surface contaminants are more loosely bound to the substrate and released first by bleach or a short pre-digestion step, leaving a higher proportion of endogenous DNA (Korlevic, et al. 2015). This is consistent with the finding that EDTA supernatants from ancient bone/tooth extracts are enriched in the pathogen *Yersinia pestis* DNA compared to pellets (Schuenemann, et al. 2011). Nonetheless, we did not observe a significant difference in the number of reads that can be classified as microbial after bleach or double digest treatments, nor a significantly different microbial profile between treatments. These latter results agree with the similarity in microbial profiles obtained from ancient horse bones that were extracted after first and second digestion (Der Sarkissian, et al. 2014). We did find a significantly higher abundance of microbial reads in Atlantic cod bones compared to those from horse. While differences in preservation conditions cannot be excluded at this stage, it is possible that the high porosity of fish bones presents a greater potential for

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microbial colonization *post-mortem*. Overall, the absence of a clear treatment effect on the microbial abundance and profile suggests that the fold-increase in authentic DNA after a bleach and short digestion treatment is not necessarily related to the (partial) removal of specific microbial contaminants.

The microbial profiles generated from the ancient cod and horse samples cluster closely to those obtained from soil, indicating that microbial contamination from humans has had limited impact on our data. Indeed, the most abundant microbial orders we observed in our samples have been previously associated with bones obtained from archaeological excavations (Der Sarkissian, et al. 2014) and likely originate from the depositional context from which these bones were retrieved. Nonetheless, some of the less abundant orders (e.g., *Marinobacter*, *Halomonas* and *Idiomarina*) have been associated with the marine environment and may also be partly associated with the host-microbiome of Atlantic cod (Star, et al. 2013) rather than the depositional context. The majority of reads in our DNA extracts remain unclassified, however, providing no further information regarding their biological origins and source.

In summary, we present a simple and cost-effective protocol to increase the endogenous DNA content of ancient bone. By combining two well-established methods, we obtained a substantial increase in the endogenous DNA content, greatly enhancing the feasibility of recovering genome-scale data in such samples.

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Acknowledgements

We are grateful to Anne-Karin Hufthammer and Jan Bill for the horse bone from the Gokstad ship burial, and we thank Vida Rajkovaca for helping with the identification of the horse bones from Orkney. This work was supported by the Research Council of Norway projects

"Tracking Viking-assisted dispersal using ancient DNA (#230821/F20 to SB), "Fisheries induced evolution in Atlantic cod investigated by ancient and historic samples" (#203850/E40), "The Aqua Genome Project" (#221734/O30), the Leverhulme Trust (to JB), the Danish Council for Independent Research, Natural Sciences (#4002-00152B) and Initiative d'Excellence Chaires d'attractivité, Université de Toulouse (OURASI to LO).

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Data accessibility

All individual read data are available at the European Nucleotide Archive (ENA, www.ebi.ac.uk/ena) under study accession number PRJEB15516.

Author contributions

SB and BS designed the study. SB, BS, KH and CDR performed the analyses. SB and BS interpreted the results with input from LO. HN and AG did the laboratory work. JHB led the excavation and identification of the Orkney samples. SB and BS wrote the paper with input from all authors.

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Figure legends

Figure 1. Overview of the experimental design. Bone powder was divided into two aliquots of which one is treated with 0.5% bleach for 15 minutes. After a short, one-hour pre-digestion, DNA was extracted from the supernatant (SD and BleSD). The pelleted bone powder was subjected to a second, overnight digestion (double digestion) using a fresh buffer, followed by DNA extraction (DD and BleDD). The protocol BleDD^2 (not shown in figure) is identical to BleDD except that twice the amount of the starting material was used, divided over two aliquots (see material and methods for details). Photograph shows specimen COD028, which was used in this study.

Figure 2. Level of endogenous DNA and clonality Atlantic cod and horse after different pre-extraction treatments. DNA was extracted from ancient Atlantic cod (COD027-COD034) and horse (VHR015-VHR064) bones, after a short pre-digestion (SD, grey), bleach combined with the short pre-digestion (BleSD, orange), a double digestion (DD, dark grey), bleach combined with a double digestion (BleDD, red) and bleach combined with a double digestion with twice the amount of input material (BleDD^2, dark red). For horse samples only DD and BleDD data were obtained. The observed clonality for BleDD of sample COD034 is an extreme outlier and not depicted to scale. Collapsed reads were down sampled to the lowest number obtained (n = 894 682).

Figure 3. Relative increase in library efficiency after bleach treatment. The fold-increase in library efficiency for BleDD (orange) and BleDD^2 (dark red) is calculated relative to the DD treatment (scaled at 1) for Atlantic cod (COD027-COD034) and horse (VHR015-VHR064). Library efficiency is measured as the fraction of high quality unique reads, after down sampling to match the lowest number of collapsed reads per bone. For horse samples only BleDD data were obtained. The dashed lines indicate the average increases for the respective treatments (BleDD and BleDD^2).

Figure 4. Length distributions of endogenous DNA templates from ancient bones. Size distributions are obtained for Atlantic cod (**a**) and horse (**b**) using the fraction of collapsed paired-end sequencing reads. DNA was extracted after a short pre-digestion (SD, grey), a bleach wash combined with the short digestion (BleSD, orange), a double digestion (DD, dark-grey), a bleach wash combined with a double digestion (BleDD, red) and a bleach wash combined with a double digestion with twice the amount of input material (BleDD^2, dark-red).

Figure 5. Microbial content of 12 ancient Atlantic cod and horse bones after different pre-extraction treatments. DNA was extracted from ancient Atlantic cod (COD027-COD034) and horse (VHR015-VHR064) bones, after a short pre-digestion (SD, grey), bleach combined with the short pre-digestion (BleSD, orange), a double digestion (DD, dark grey), bleach combined with a double digestion (BleDD, red) and bleach combined with a double digestion with twice the amount of input material (BleDD^2, dark red). For horse samples only DD and BleDD data were obtained. Reads were classified at the genus level with metaBIT, aligning reads to the MetaPhlAn2 database.

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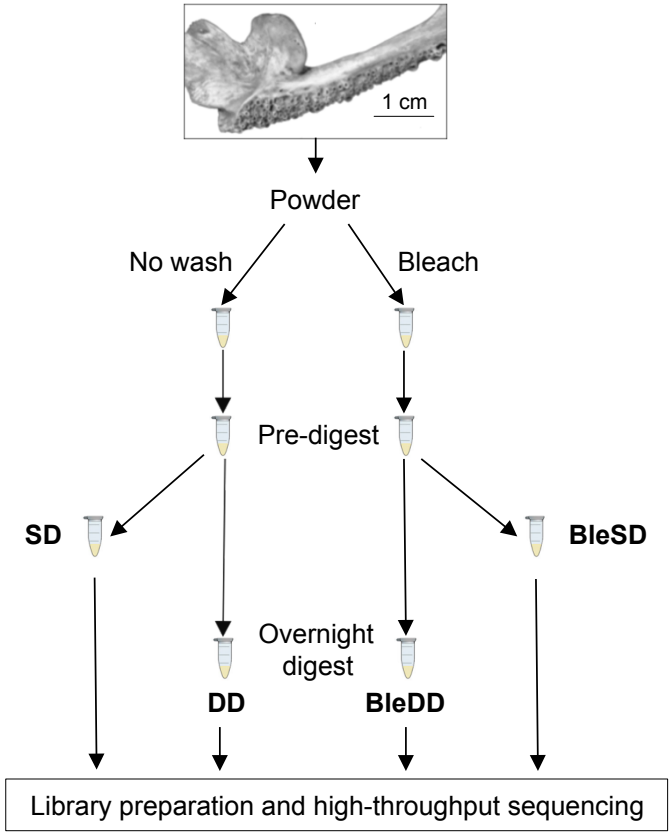
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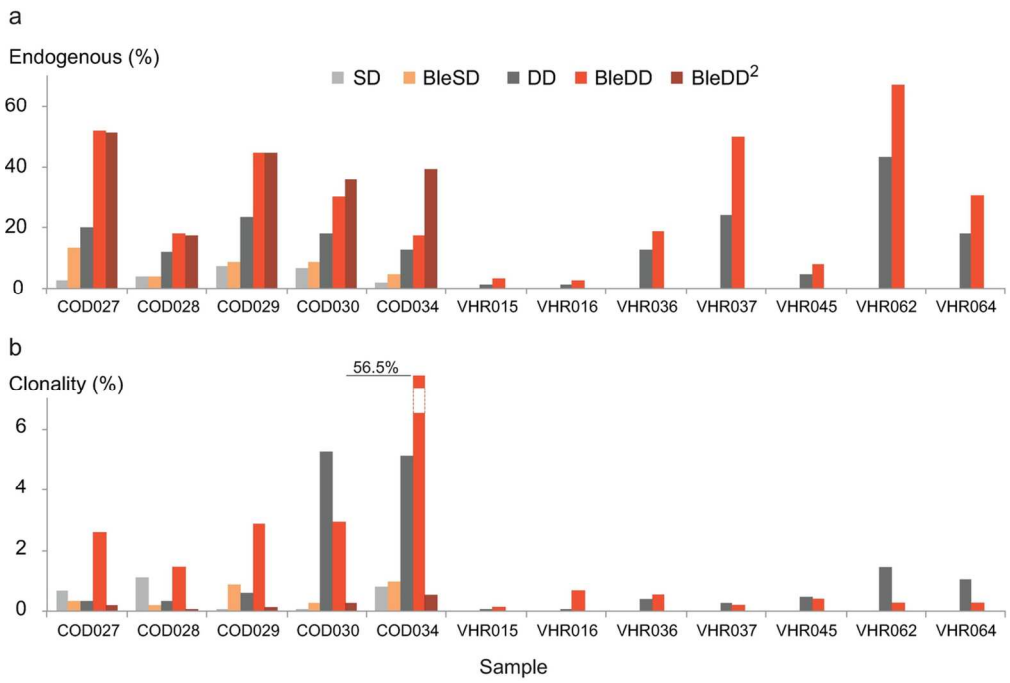

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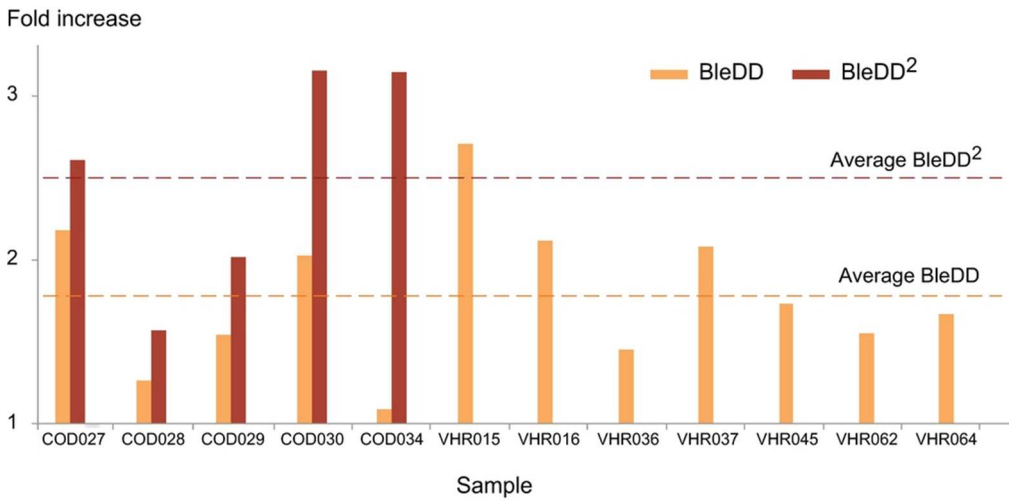

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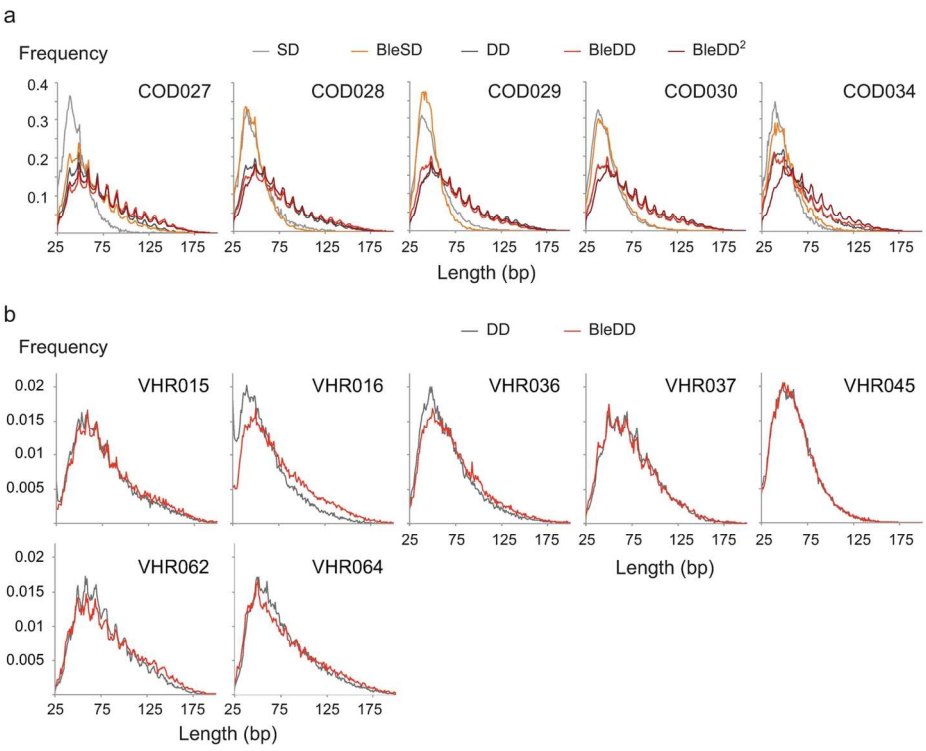

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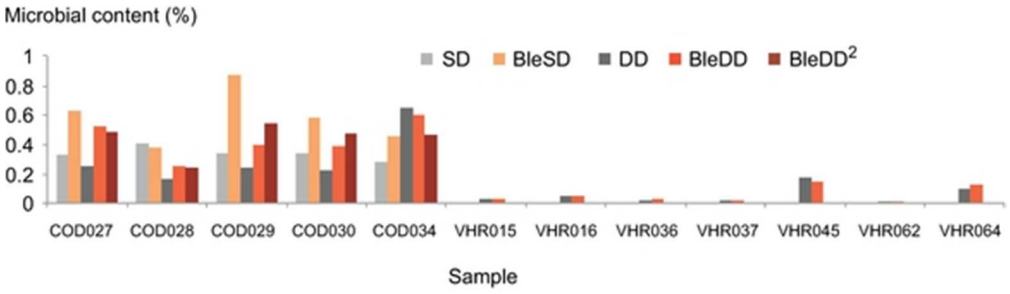

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