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Benchmark

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Comparison and optimization of protocols and whole-genome capture conditions for ancient DNA samples

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ABSTRACT

Ancient DNA (aDNA) obtained from human remains is typically fragmented and present in relatively low amounts. Here we investigate a set of optimal methods for producing aDNA data by comparing silica-based DNA extraction and aDNA library preparation protocols. We also test the efficiency of whole-genome enrichment (WGC) on ancient human samples by modifying a number of parameter combinations. We find that the Dabney extraction protocol performs significantly better than alternatives. We further observed a positive trend with the BEST library protocol indicating lower clonality. Notably, our results suggest that WGC is effective at retrieving endogenous DNA, particularly from poorly-preserved human samples, by increasing human endogenous proportions by 5x. Thus, aDNA studies will be most likely to benefit from our results.

METHOD SUMMARY

We studied a set of methods used to generate ancient genome data from archaeological human samples from different sites and periods, and with varying levels of preservation. In these experiments we compared two sample preparation techniques, two silica-based DNA extraction protocols, and two aDNA library preparation protocols. Finally, we explored the efficiency of whole-genome capture (WGC) on aDNA libraries with different characteristics by comparing a set of protocol modifications.

KEYWORDS:

ancient DNA • DNA extraction • aDNA library • whole genome capture • optimization

Ancient DNA (aDNA) data obtained from archaeological remains an expanding and well-established field that has already helped resolve various questions in evolutionary biology and anthropology. However, aDNA also exhibits significant technical challenges. For example, authentic aDNA is retrieved as damaged and degraded into short fragments, and mixed with large amounts of bacteria and other environmental DNA [1–3]. These properties impact the efficiency of shotgun sequencing of ancient genomes. Although high-throughput sequencing and in-solution capture techniques have helped to partly overcome these challenges, most archaeological samples still remain too badly preserved for in-depth genomic analysis. Over the last decade, a number of new approaches have mitigated these issues, i.e., improvements in DNA extraction and library preparation [4–6]. But obtaining sufficient authentic aDNA still remains a challenge, and this particularly holds true for samples from temperate, warm, humid areas, and acidic soils [7].

Hybridization-based capture approaches are explored to increase the data yield and decrease the cost. The most frequently used is targeted single nucleotide polymorphism (SNP) capture; although promising, it relies on pre-ascertained SNP data. This poses disadvantages over analyzing whole-genome shotgun data by limiting the set of statistical analyses applicable to genomic data and by suffering from downstream biases. Therefore, a whole-genome capture (WGC)-based method could be a promising alternative [8,9]. Recently, WGC has been used to generate genomic data from Neolithic and Bronze Age samples [10,11]. However, the popularity of WGC seems to have declined lately, mainly because of high clonality levels observed in these libraries. To our knowledge, though, no systematic WGC optimization trial has yet been performed on samples from various periods with various preservation levels.

Here, we aimed to develop an optimized set of methods to generate genomic data from poorly preserved human samples, building on the original protocols. We studied three stages of aDNA data production: DNA extraction, library preparation and whole-genome enrichment (Figure 1). We used archaeological samples from different sites and periods, especially from warm areas, and with varying levels of preservation. We compared two sampling methods, drilling versus freezer-milling; two protocols for silica-based aDNA extraction, Ottoni *et al.* [12] versus Dabney *et al.* [13]; and two library preparation protocols, BEST [14] versus M&K [15]. We also explored different conditions used for a WGC approach and thus optimized DNA enrichment on ancient human samples. We used five sequencing summary statis-

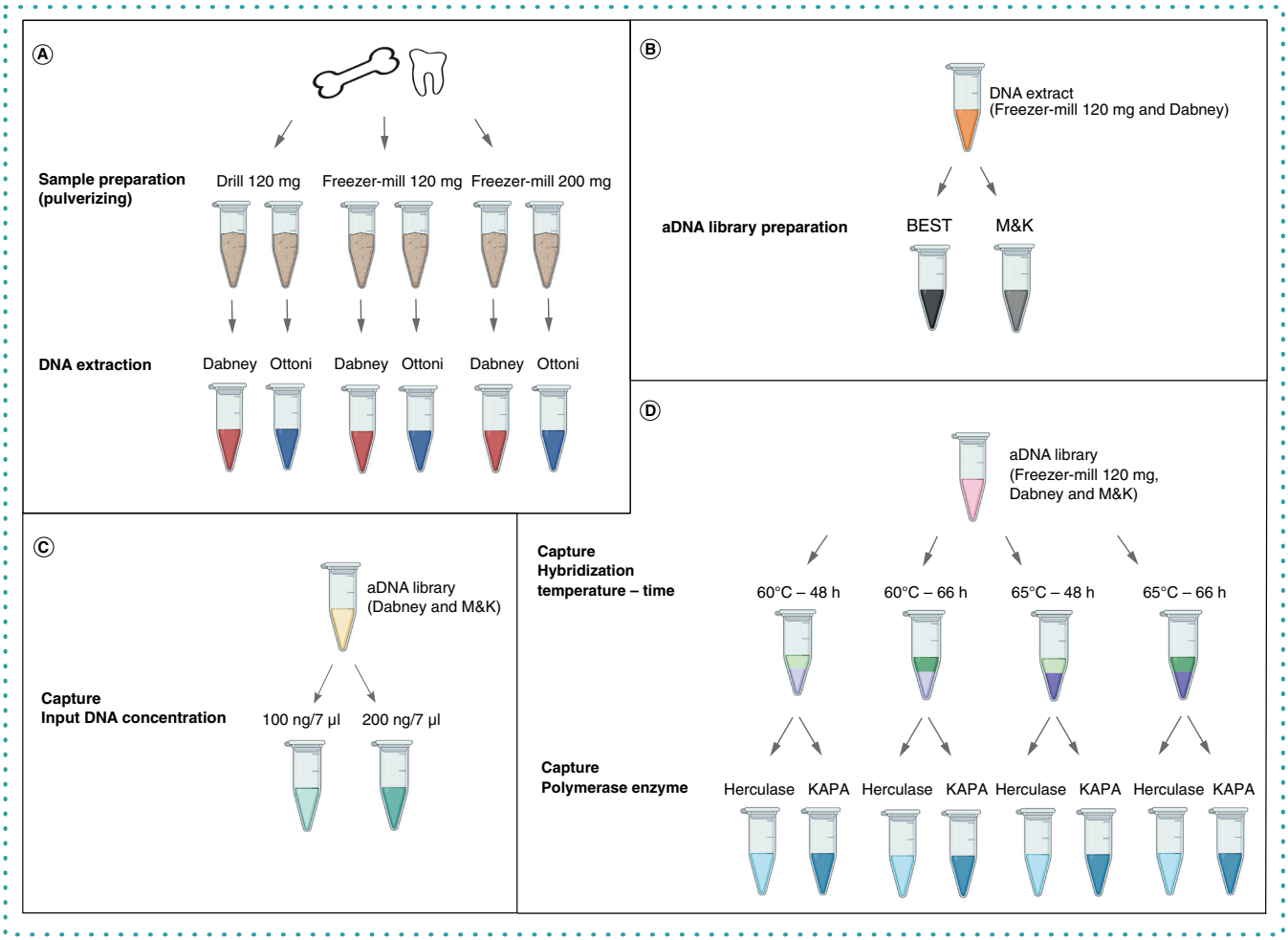

Figure 1. Experimental design. (A) For the comparisons of sample preparation and DNA extraction methods. This was applied to n = 2 samples. **(B)** For the comparisons of aDNA library protocols: BEST and M&K. This was applied to n = 27 samples. **(C)** For the input DNA concentration tests used in whole-genome capture. This was applied to n = 3 samples. **(D)** For the comparisons and optimization of the whole-genome capture conditions. This was applied to n = 15 samples.

tics for all comparisons as evaluators: endogenous human DNA proportion (excluding duplicate reads), clonality, average read length, GC-content, and the normalized number of SNPs covered at least once (Supplementary Appendix 1).

We first compared sample preparation (pulverizing) techniques and extraction protocols, exploring different combinations and using n = 2 samples. We find that the Dabney [13] DNA extraction protocol performs significantly better compared to the Ottoni [12] protocol with respect to human DNA proportion (median = 3.7× higher with Dabney), clonality and number of SNPs (Wilcoxon signed-rank test [WSRT] $p < 0.05$; Figures 2 & 3A & Supplementary Figure 1). We observed no significant difference between two protocols based on average read length (WSRT p = 0.69; Supplementary Table 9). GC-content of sequenced reads was on average higher using Ottoni (Dabney median = 38.7%; Ottoni median = 45.1%), which might be related to the higher clonality. Our results further suggest that freezer-milling is a better technique than drilling for sample preparation (Figure 3B & Supplementary Figure 1), which is consistent with an earlier study [16]. Although not significant, there is a positive trend toward higher yields with freezer-milling (WSRT $p > 0.1$; Supplementary Table 9). However, we also note that the observed difference in performance between freezer-milling and drilling reported in this study could be minimized by using even lower drilling speeds, as reported in Adler *et al.* [16].

We next compared two aDNA library preparation protocols, M&K [15] and BEST [14], using n = 27 samples from various archaeological periods (Supplementary Appendix 1). Our results showed nonsignificant differences between the BEST and M&K protocols when all samples (n = 27) were considered with respect to human DNA proportion, average read length, GC-content and number of SNPs. However, the BEST protocol resulted in significantly lower clonality (WSRT $p < 0.0001$; BEST median = 18.2%; M&K median = 26.6%). Also, BEST showed a positive trend with respect to both human DNA proportion and number of SNPs, although nonsignificant (Figure 4A & Supplementary Figure 1 & Supplementary Table 9). Likewise, we observed the same pattern in within-period comparisons (e.g., poorly preserved early Neolithic samples [n = 17]) for clonality, human DNA proportion and number of SNPs, as we did in all-sample compar-

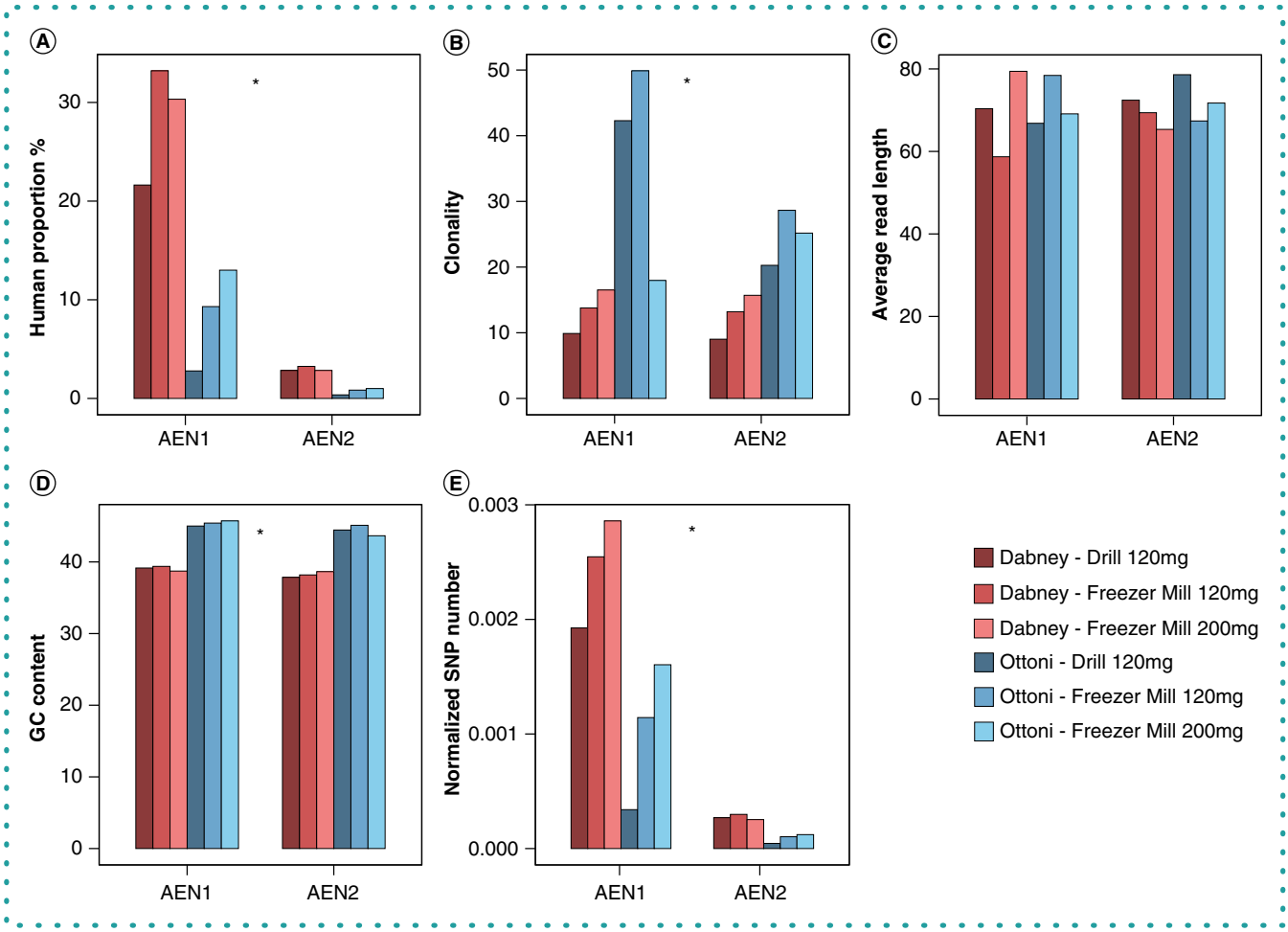

Figure 2. Five summary statistics of the sequence data obtained by applying each combination of pulverization method and extraction protocol to two samples: AEN1 and AEN2. (A) Human DNA proportion (%). **(B)** Clonality. **(C)** Average read length. **(D)** GC-content (%). **(E)** Number of single nucleotide polymorphisms. The significant results are marked with one asterisk ($p < 0.05$); 120 mg and 200 mg sample powder obtained using drill and freezer-mill.
Dabney: Dabney *et al.*'s aDNA extraction protocol; Ottoni: Ottoni *et al.*'s aDNA extraction protocol.

isons (Figure 4B, Supplementary Figure 2 & Supplementary Table 9). Our interpretation is that BEST causes lower DNA loss, most likely due to the single tube technique and fewer cleanup steps involved in the protocol. We also suggest that the lower clonality of BEST libraries, when combined with the relatively higher human DNA content, allows for deeper sequencing.

We further investigated the performance of WGC on aDNA libraries using the myBaits Human Whole Genome Capture Kit (Arbor Biosciences, MI, USA). We aimed to identify the parameter settings that provide the most desirable results for WGC on aDNA using samples with varying levels of preservation. We applied a set of modifications to the WGC parameters given in the aforementioned kit protocol (Supplementary Appendix 1 & Figure 1). To assess the performance of WGC, we compared the same five summary statistics of the enriched libraries with the screening results from the same libraries (Supplementary Tables 7 & 8). Across all conditions and libraries (n = 126 tests), we observed modest increases after the enrichment in both human DNA proportion and number of SNPs (0.3×–60.4×, median = 2.8×, and 0.06×–48×, median = 1.5×, respectively; Figures 5–7 & Supplementary Figure 5).

We first tested the effect of input DNA concentration levels, 100 ng/7 μl versus 200 ng/7 μl, using a library set (n = 3) from different regions and periods (Supplementary Appendix 1). We found a trend toward better performance with 200 ng/7 μl, with a slight difference from 100 ng/7 μl (Figure 5). However, the sample size was not sufficient to show significance, which may turn out to be replicable with larger sample sizes.

We next studied the effects of six parameter settings and their different combinations on the efficiency of aDNA WGC: two different polymerases used at the amplification step of the enriched libraries: KAPA HiFi HotStart Polymerase (Kapa Biosystems) and Herculase II Fusion DNA Polymerase (Agilent Technologies), two hybridization temperatures (60°C and 65°C) and two hybridization times (48 h and 66 h). The set of libraries (n = 15) was chosen according to diverse values for human DNA proportion statistics including various

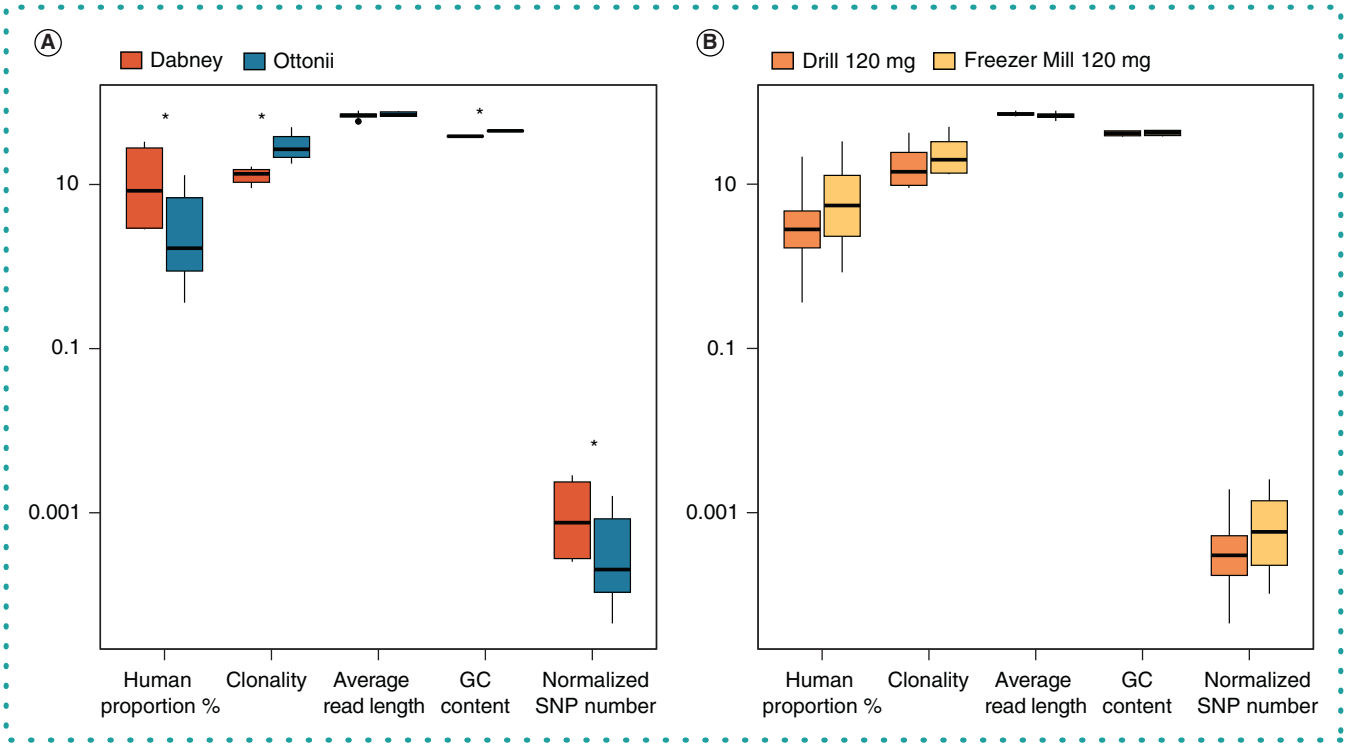

Figure 3. Boxplots show comparisons of the methods with respect to five summary statistics obtained from the sequence data. (A) For the two extraction protocols. **(B)** For the pulverization techniques (drill vs freezer-mill) using 120 mg bone powder as starting material (supplementary data). The significant results are marked with one asterisk ($p < 0.05$). The y-axes are log scaled.

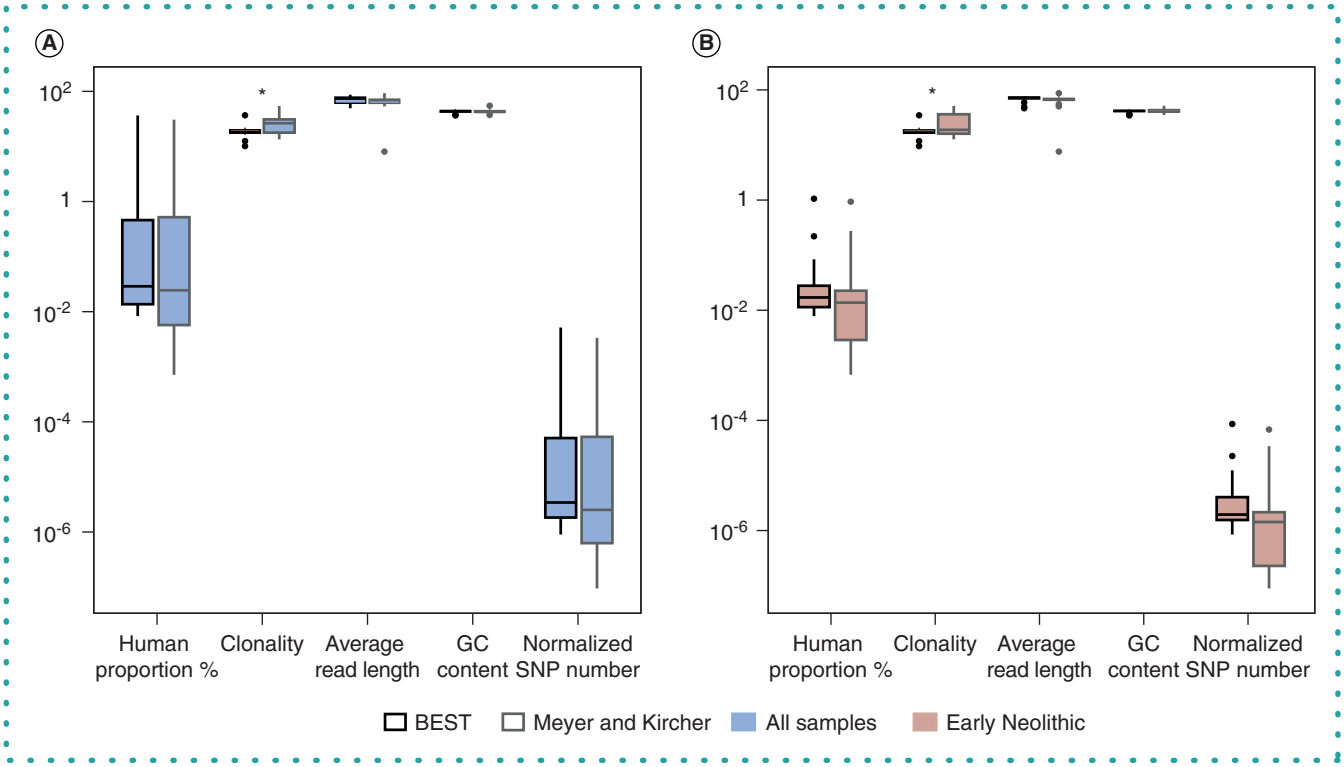

Figure 4. Boxplots show comparisons of the BEST and M&K protocols with respect to five summary statistics obtained from the sequence data. (A) All-sample comparisons from varying periods (n = 27). **(B)** Poorly preserved samples from the early Neolithic period (n = 17). The significant results are marked with one asterisk ($p < 0.05$). The y-axes are log scaled.

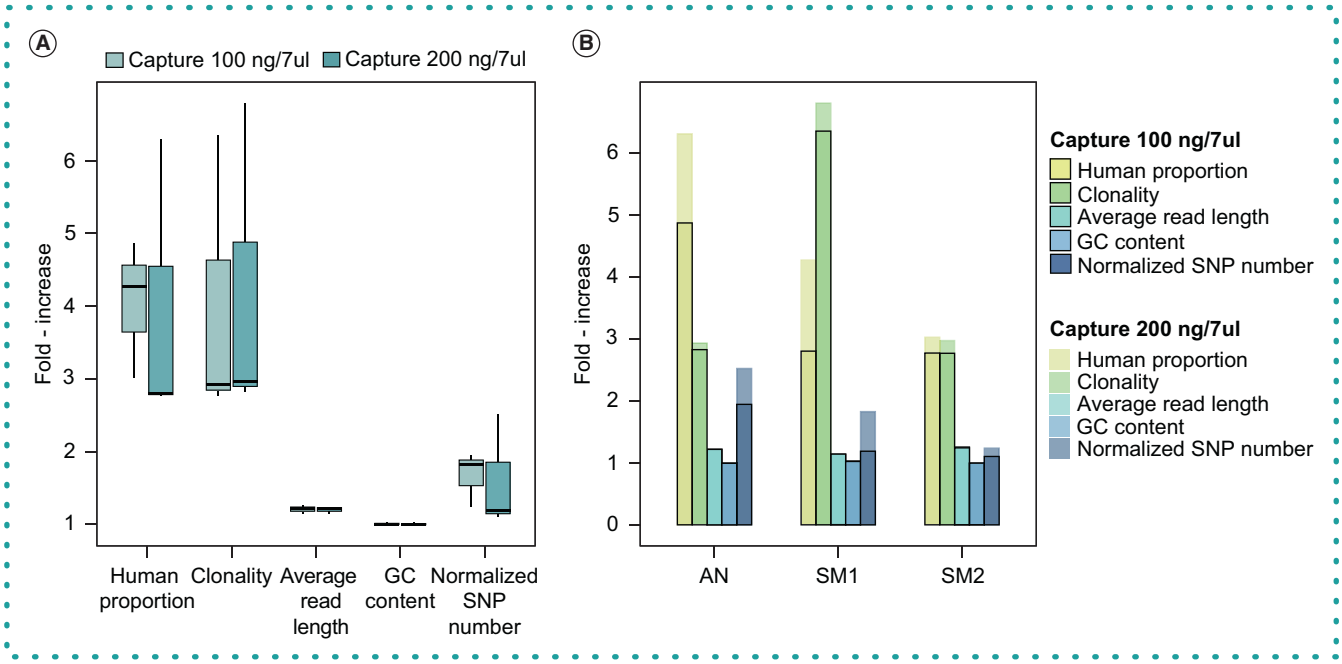

Figure 5. Five summary statistics of the whole-genome enriched libraries using 100 ng/7 μl and 200 ng/7 μl input DNA concentrations. Fold-increase shown on the y-axis indicates an increase in the capture compared with the shotgun sequencing data (precapture) for **(A)** all-sample level and **(B)** per sample level.
AN: Anatolian Neolithic; SM: Scandinavian Medieval

combinations (e.g., low human proportion with high clonality; Supplementary Table 8). Libraries were also chosen to represent DNA from five archaeological periods, such that at least two samples from each period were included (Supplementary Appendix 1).

First, we compared two different polymerases at the amplification step after the enrichment. We found that Herculase was more efficient than KAPA with respect to human DNA proportion, clonality, average read length and number of SNPs (WSRT $p < 0.01$; Figure 6 & Supplementary Table 9).

Second, when comparing hybridization temperatures (60°C and 65°C), we found that 60°C yielded a significantly higher human DNA proportion and number of SNPs relative to 65°C (WSRT $p < 0.0001$). However, the difference in clonality, average read length and GC-content between the two hybridization temperatures was nonsignificant (WSRT $p > 0.1$; Figure 6 & Supplementary Table 9).

Third, when comparing hybridization time (48 h and 66 h), we found that the libraries incubated 66 h yielded significantly lower clonality compared with the libraries incubated 48 h (WSRT $p < 0.01$). Although our results present a positive trend toward the 66-h hybridization time, we found no significant difference with respect to human DNA proportion, average read length, GC-content and number of SNPs (Figure 6 & Supplementary Table 9).

Finally, we compared the four parameter combinations of hybridization temperature (60°C and 65°C) and time (48 h and 66 h). We found that the combination of 60°C/66 h generated better results relative to the alternatives. This combination produced significantly lower clonality and higher number of SNPs than 60°C/48 h (WSRT $p < 0.001$). Here, we also observed the same pattern based on human DNA proportion, but nonsignificantly (60°C/66 h median = 0.02; 60°C/48 h median = 0.014; WSRT $p > 0.5$). Besides, 60°C/66 h yielded significantly better results than both 65°C/66 h and 65°C/48 h with respect to human DNA proportion and number of SNPs (WSRT $p < 0.001$ and $p < 0.01$, respectively), while there was no significant difference concerning other summary statistics: clonality, average read length and GC-content. Our results suggest that the combination of 60°C/66 h results in a higher number of SNPs across all combinations (WSRT $p < 0.05$). Furthermore, we did not observe any significant difference between the 65°C/66 h and 65°C/48 h combinations (Figure 6 & Supplementary Table 9).

Our results show that the WGC approach can increase the sequencing efficiency, leading to significant increase in both human DNA proportion and number of SNPs after enrichment. We also note that WGC on aDNA could work even with lower input DNA concentration (100 ng/7 μl) and lower human DNA proportions (<1%). Regarding hybridization temperature and time, 60°C/66 h was identified as an optimal combination. This combination provides more opportunities for hybridization, suggesting the main constraint is not background noise but human DNA annealing. Thus, future optimization efforts could investigate the effect of different combinations (e.g., longer hybridization and lower temperatures) than those recommended.

We further find that a precapture human DNA proportion of 1–5% led to modest enrichment in both human DNA proportion and number of SNPs (2.5×–5× and 1.4×–2.9×, respectively; Figure 7 & Supplementary Figures 4 & 5). However, human DNA proportion and

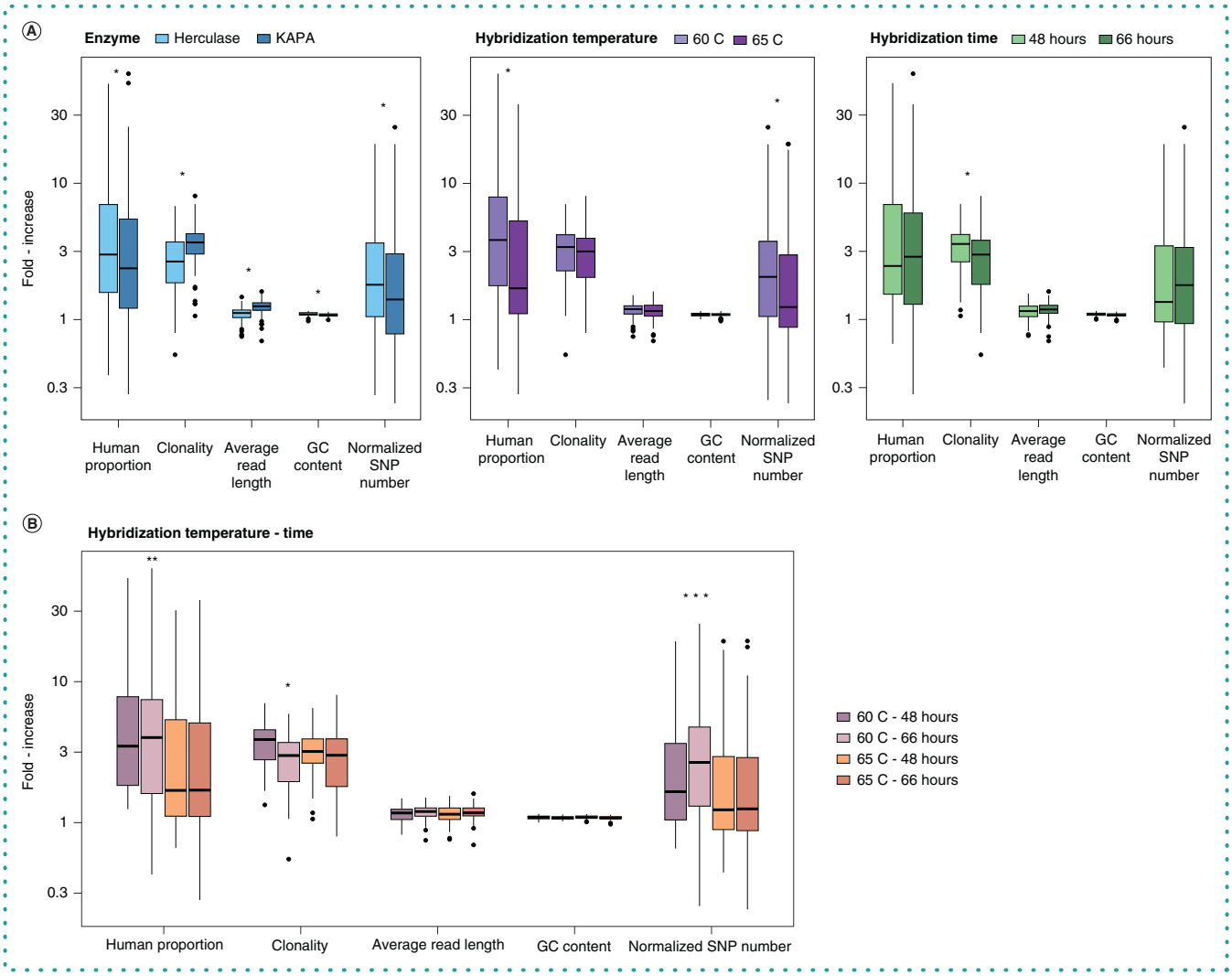

Figure 6. Relative efficiency of the whole-genome-enriched libraries with respect to five summary statistics. (A) Using the two polymerase enzymes (Herculase and KAPA), two hybridization temperatures (60°C and 65°C) and two hybridization times (48 h and 66 h), respectively. Statistically significant results are marked with one asterisk ($p < 0.05$). **(B)** Using different combinations of hybridization temperature and time (60°C/48 h, 60°C/66 h, 65°C/48 h and 65°C/66 h). Statistically significant results ($p < 0.05$) are marked with asterisks: one asterisk for the comparisons of 60°C/66 h versus 60°C/48 h, two asterisks for the comparisons of 60°C/66 h versus 65°C/48 h and 65°C/48 h and three asterisks for the comparisons of 60°C/66 h versus all three other combinations. The y-axes are log scaled.

number of SNPs could be enriched between 4.4×–35× (median = 9.6×) and 3×–18.4× (median = 5.8×), respectively, within reasonable clonality levels (21.1–70.5%), for the libraries with even lower precapture human DNA proportion (0.01–1%; Figure 7 & Supplementary Figure 5). This contrasts with earlier studies that reported that a precapture DNA content of 1–25% was needed to result in effective enrichment [17,18]. We also find that overall WGC increases clonality, average read length and GC-content to a low extent, as observed in previous studies [9,18–20].

Overall, our results suggest that WGC is an efficient and cost-effective method for retrieving endogenous human DNA in samples of various preservation levels. Besides, this method, in contrast to targeted SNP capture, has the potential to generate unbiased data and, thereby, could present a viable option in future aDNA research targeting low-quality libraries.

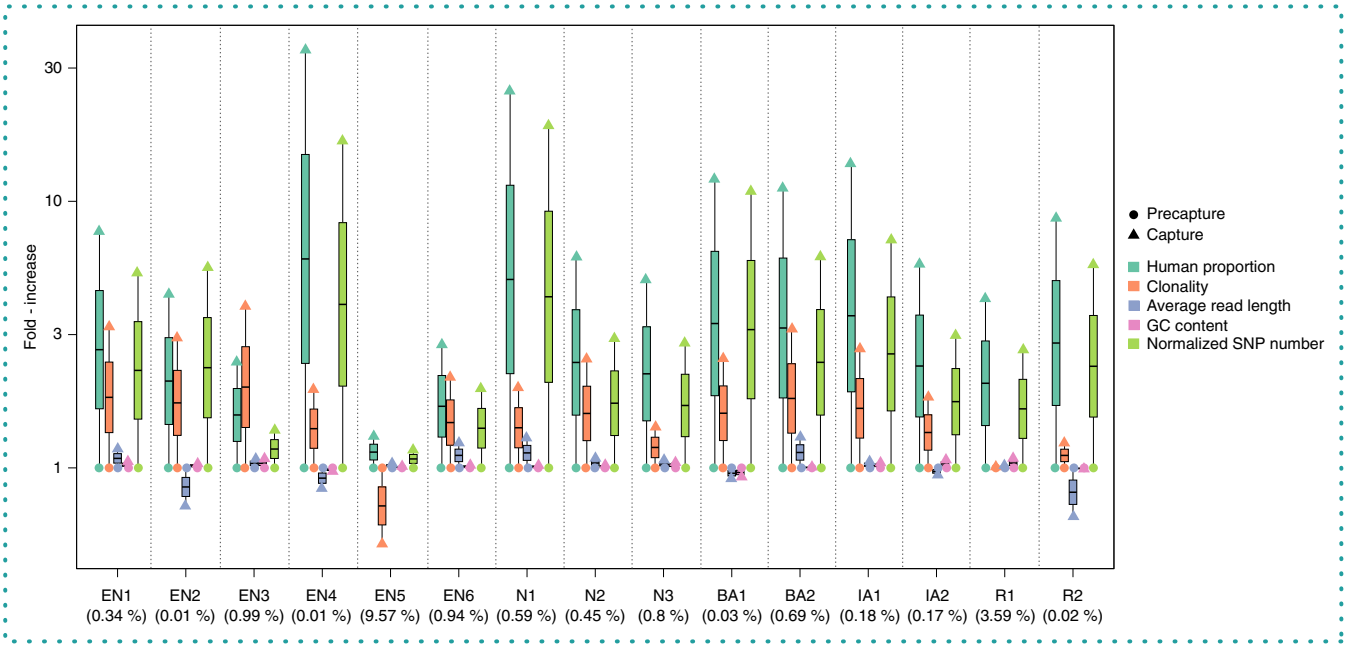

Figure 7. Five summary statistics of each sample before and after the enrichment concerning the best combination across all the parameter combinations for each sample. The best result for each sample was chosen across n = 8 combinations (two polymerase enzymes [Herculase vs KAPA], two hybridization temperatures [60°C vs 65°C] and two hybridization times [66 h vs 48 h]). Precapture human DNA proportion of each library is given in parentheses. The y-axis is log scaled.
BA: Bronze Age; EN: Early Neolithic; IA: Iron Age; N: Neolithic; R: Roman Period.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2023-0107

Author contributions

R Yaka, VK Lagerholm and M Krzewinska designed the study with contributions by F Özer, M Somel and A Götherström; R Yaka and VK Lagerholm performed laboratory work; R Yaka conducted data analysis; R Yaka, M Krzewinska, VK Lagerholm, A Linderholm, F Özer, M Somel and AG wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, stock ownership or options and expert testimony.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

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• **Excellent comparative study regarding whole-genome capture method on ancient DNA.**