Comparison of two ancient DNA extraction protocols for skeletal remains from tropical environments

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ABSTRACT

Objectives: The tropics harbor a large part of the world's biodiversity and look back on a long history of human habitation. However, paleogenomics research in these climates has been constrained so far by poor ancient DNA yields. Here we compare the performance of two DNA extraction methods on ancient samples of teeth and petrous portions excavated from tropical and semi-tropical sites in Tanzania, Mexico, and Puerto Rico (N=12).

Materials and Methods: All samples were extracted twice, built into double-stranded sequencing libraries, and shotgun sequenced on the Illumina HiSeq. The first extraction protocol, Method D, was previously designed for recovery of ultrashort DNA fragments from skeletal remains. The second, Method H, modifies the first by adding an initial EDTA wash and an extended digestion and decalcification step.

Results: No significant difference was found in overall ancient DNA yields, number of unique sequence reads, endogenous DNA content, or post-mortem damage patterns recovered from samples extracted with either method, irrespective of tissue type. However, samples extracted with Method D have shorter average DNA fragments and higher GC content.

Discussion: Both methods successfully recovered endogenous ancient DNA. But, since surviving DNA in ancient or historic remains from tropical contexts is extremely fragmented, our results suggest that Method D is the optimal choice for working with samples from warm and humid environments. Additional optimization of extraction conditions and further testing of Method H with different types of samples may allow for improvement of this protocol in the future.

Ancient DNA (aDNA) is a low quality and low quantity source of genetic material, which is highly susceptible to external contamination (Gilbert et al., 2006; Hofreiter et al., 2001; Pääbo, 1989). Due to the variety of taphonomic and diagenetic processes that take place after death, DNA decays exponentially once cell repair functions cease in biological tissues (Hofreiter et al., 2001). Consequently, most genetic information obtained from ancient samples is contained in small, degraded DNA fragments (Allentoft et al., 2012; Briggs et al., 2007; Dabney et al., 2013a). Because of this, until recently, aDNA studies have focused on short but informative fragments of the autosomal genome or, alternatively, on multicopy loci such as mitochondrial DNA (Ho & Gilbert, 2010). Recent advances in DNA extraction, target enrichment, and next-generation sequencing methods now allow the recovery of complete genomes from remains dating as far back in time as the early Holocene and Middle Pleistocene (Meyer et al., 2016; Meyer et al., 2014; Orlando et al., 2013).

Despite these improvements in stretching the time depth for aDNA recovery, paleogenomics research continues to be constrained in its geographic focus because DNA preservation is negatively correlated with thermal age due to the accelerating effect of high temperatures on biomolecule decay and fragmentation (Adler et al., 2011; Allentoft et al., 2012; Hofreiter et al., 2015; Kistler et al., 2017; Lindahl, 1993; Smith et al., 2001). Therefore, most aDNA studies focus on archaeological remains excavated from cold and temperate world regions, which have the highest chance of DNA survival (Paijmans et al., 2013; Wade, 2015). Nevertheless, despite the challenges of working with poorly preserved samples, several studies have successfully recovered aDNA from tropical sites in the Caribbean, the Yucatan peninsula and South-East Asia (Damgaard et al., 2015; Gamba et al., 2014; Gutierrez-Garcia et al., 2014; Kehlmaier et al., 2017; Mendisco et al., 2015; Schroeder et al., 2015).

Both today and in the past, the tropics harbor a large part of the world’s biodiversity and human settlements (Brown, 2014; Buzas et al., 2002). Therefore, understanding how DNA is preserved in degraded remains excavated from these environments and optimizing or improving methods that facilitate aDNA recovery from these contexts is of great interest to advance anthropology, paleontology, and conservation genetics, among other fields.

Here we test the performance of two DNA extraction protocols on tooth and petrous portion samples from degraded skeletal remains recovered from tropical sites in Tanzania, Mexico and Puerto Rico (N=12). Specifically, we compare the method developed by (Dabney et al., 2013a), hereafter Method D, to a second approach, Method H. Method H modifies the former by adding an initial EDTA wash, as in Warinner et al. (2014), and an extended digestion and decalcification step as in Gamba et al. (2016). Method D was specifically designed to increase recovery of extremely short DNA fragments (as small as 30 base pairs) in ancient bone and tooth extractions. In line with earlier protocols (Höss & Pääbo, 1993; Rohland & Hofreiter, 2007), this method employs a 24-hour proteinase K digestion to break up cell proteins, and uses a chaotropic guanidium-based salt to bind DNA fragments and remove inhibitors. It differs from previous approaches in its use of silica spin columns and a guanidine hydrochloride binding buffer (instead of guanidine thiocyanate). Method D has been successfully employed in the recovery of aDNA from Late Pleistocene cave bear remains (Dabney et al., 2013a), of Middle Pleistocene hominin fossils (Meyer et al., 2016; Meyer et al., 2014), and of a large variety of more recently dated human and animal remains, including at least one from a tropical context (Günther et al., 2015; Heintzman et al., 2015; Kehlmaier et al., 2017; Seguin-Orlando et al., 2014).

Since Method D was developed, several extraction protocol modifications have been proposed for improving endogenous aDNA recovery. Comparing different extraction methods, Gamba et al. (2016) found that a secondary digestion and decalcification using lysis buffer with EDTA, proteinase K and N-laurylsarcosyl detergent solution, aided in solubilizing cell proteins and resulted in increased aDNA yields. Similarly, Damgaard et al. (2015) observed that a brief pre-digestion (between 15 and 30 minutes) with an EDTA and proteinase K buffer was successful in reducing proportions of exogenous, contaminant DNA and in enriching extracts for endogenous aDNA. The use of similar detergent solutions has been implemented previously in extraction protocols designed by Richards et al. (1995) and was also recently reported in extractions of petrous portion tissue (Gamba et al., 2014; Pinhasi et al., 2015). Likewise, Warinner et al. (2014) used an initial EDTA wash to remove loosely bound surface contaminants on mineralized dental calculus without significant DNA loss. This finding was mirrored by Tromp et al. (2017) who observed that EDTA decalcification was more effective at recovering microparticles from dental calculus than hydrochloric acid.

In this study, we evaluate whether using a modified version of the Method D protocol (henceforth Method H), with an initial EDTA wash and an extended digestion and decalcification step, results in improved endogenous aDNA recovery. In addition, because the majority of development in aDNA extraction protocols has been conducted with samples from temperate or cold contexts (Barlow et al., 2016; Boessenkool et al., 2016; Dabney et al., 2013a; Gamba et al., 2016; Gamba et al., 2014; Glocke & Meyer, 2017) but see (Damgaard et al., 2015; Pinhasi et al., 2015; Tromp et al., 2017), this work focuses on protocol optimization with tooth and petrous portion samples recovered from tropical sites. Here we examine raw DNA yields and endogenous reads, recovered after shotgun Illumina sequencing from parallel, paired extractions and characterized differences in base pair composition, post-mortem damage profiles, and average read lengths recovered between the two methods. Archaeological samples included in this research were obtained from human remains excavated at three Ceramic Age sites from Puerto Rico (n=5) and one tomb from the Maya site of Yaxuna in Yucatán, Mexico (n=6). Additionally, one historic sample from a Tanzanian chimpanzee was also included.

Study results suggest that both methods were similarly efficient at aDNA recovery. But libraries sequenced from Method D extracts have higher proportions of shorter DNA fragments and higher average GC content. Since most of the archaeological samples had extremely low endogenous content (<1%), and average DNA fragment sizes were under 80 bp, we conclude that method D is better suited than Method H for maximized recovery of informative ancient DNA molecules from remains buried in tropical environments. However, one important caveat of our study is that these findings are only applicable to tooth samples, since the small sample of petrous portions obtained led to inconclusive results in statistical tests conducted with this tissue.

MATERIALS AND METHODS

Sample and site information

Samples were collected from skeletal remains excavated from three tropical contexts. Tissue samples from petrous portions and/or teeth were obtained from six human skeletons excavated from a single tomb in the archaeological site of Yaxuna in Yucatán, Mexico. This was an originally unfilled burial space that dates to the Maya Early Classic period (specifically from the 6th century A.D.) During the centuries of deposition, the skeletonized remains gradually filled with rubble and fill falling from the ceiling of the chamber. In most skeletons, only one tissue type, petrous portion or teeth was available for sampling, while only two individuals (AD-372 and AD-373) could be sampled in both anatomic locations (Table 1). Additionally, five teeth were collected from humans remains excavated from three open-air sites in Puerto Rico: Tibes (n=1), Paso del Indio (n=2), and Punta Candelero (n=2). All five individuals date from pre-contact Ceramic Age contexts, between A.D. 500-1300 (Pestle, 2010; Pestle & Colvard, 2012). Lastly, one tooth was collected from the skeletal remains of a wild chimpanzee (*Pan troglodytes schweinfurthii*) who died of natural causes in 1966 and was buried at Gombe Stream National Park in western Tanzania (filled burial environment). Petrous portion tissue was not available from the Puerto Rican or Tanzanian remains. In total, 12 individual skeletons were sampled producing ten teeth and four petrous portions.

[Table 1 here]

Sample processing and DNA extraction

Sample processing and DNA extractions were conducted at the Arizona State University Ancient DNA Laboratory, a Class 10,000 clean-room facility. To eliminate surface contaminants and inhibitors, tooth and bone samples were cleaned with a 1% sodium hypochlorite solution. The outer surface was mechanically removed with a Dremel tool (Rohland & Hofreiter, 2007) and samples were UV irradiated for 5 minutes on each side in a UVP CL-1000 Ultraviolet Crosslinker. Teeth were sliced transversally at the cemento-enamel junction using a Dremel tool. The roots were covered in aluminum foil and pulverized by blunt force with a hammer as in Schuenemann et al. (2011). Petrous portions were sampled as recommended by Pinhasi et al. (2015). All laboratory procedures were conducted using contamination controls, such as use of full body coverings, bleach decontamination and UV irradiation of tools and work area before and between uses (Cooper & Poinar, 2000; Gilbert et al., 2006).

Each sample was extracted twice, one time using Method D and a second time using Method H. Method D was implemented as in (Dabney et al., 2013a) with the modification that the TET buffer was warmed to 65 °C in a heat block. Method H combines steps from earlier protocols including an initial EDTA wash as in Warinner et al. (2014), an extended digestion and decalcification step as in Gamba et al. (2016), and binding and purification steps as in (Dabney et al., 2013a). See Supplementary File S1 for complete Method H protocol. Approximately 100 mg of bone or tooth powder were used for each extraction. 1 µl of each extract was used to measure DNA yields through fluorometric quantification with the Qubit 2.0 High Sensitivity assay (Table S1) (Simbolo et al., 2013). Extraction blanks were included throughout the process to monitor potential contamination.

Library preparation and sequencing

Double stranded libraries were built following the protocol by Meyer and Kircher (2010) and including negative controls. DNA content in the libraries was quantified using real-time PCR (qPCR) with the 2X Dynamo SYBR Green qPCR Master Mix to determine the ideal amount of indexing cycles. All libraries were double indexed and amplified for 11-25 cycles following published guidelines (Kircher et al., 2012; Seguin-Orlando et al., 2015). Indexed libraries were purified using the Qiagen MinElute PCR purification kit and DNA content after amplification was determined via qPCR using the KAPA Library Quantification kit following manufacturer instructions (Kapa Biosystems). Fragment analysis of the indexed libraries was conducted with the DNA 1000 assay on the Agilent 2100 Bioanalyzer. Heteroduplexes that arose during indexing were eliminated through reconditioning PCR and all libraries were purified and re-quantified as detailed above. Reconditioned shotgun libraries were sequenced on one lane across two runs on the Illumina HiSeq 2500 (2 x 100 bp reads) at the Yale Center for Genomic Analysis. See Supplementary File S1 for additional details of library preparation, PCR primers and conditions.

Shotgun read mapping and processing

Illumina sequence reads were merged and adapters trimmed using SeqPrep (https://github.com/jstjohn/SeqPrep). Sequence reads were randomly selected for each sample-treatment combination to match the lowest number of reads obtained per sample using seqtk (https://github.com/lh3/seqtk) (Table S2). For the human samples, the down sampled reads were mapped to the GRCh37 (hg19) reference with the mitochondria replaced by the revised Cambridge Reference Sequence (rCRS) (Andrews et al., 1999). For the chimpanzee samples, the reads were mapped to the PanTro4 assembly. Mapping was performed using BWA v. 0.7.5 (Heng Li & Durbin, 2009) following recommendations by Schubert et al. (2014). Quality filtering (≥ Q30), removal of duplicates and of reads with multiple mappings was performed with SAMtools v. 0.1.19 (H. Li et al., 2009). BAM files were rescaled and damage patterns were characterized using mapDamage v.2.0.2 (Ginolhac et al., 2011; Jónsson et al., 2013). Those parameters examined included deamination patterns, probability of C to T misincorporations at first position, probability of G to A misincorporations at last position, probability of a DNA fragment terminating in a single-stranded overhang (λ), probability of observing cytosine deamination in a double strand (δ_D), and probability of observing cytosine deamination in a single strand context (δ_S). Library complexity estimates were generated using *preseq* v2.0 (Daley & Smith, 2013) on downsampled BAM files containing all Q30 mapped reads. Future experiment yield predictions were calculated extrapolating to 1e+10 total reads. Summary statistics were estimated on rescaled BAM files using Qualimap v.2.2.1 (Okonechnikov et al., 2016). See Supplementary File S2 for additional details of shotgun sequence read processing.

Statistical Analyses

Extraction yields (ng/µL), number of mapped, unique reads, percent endogenous content, library complexity (measured as percent distinct reads), percent GC content, average fragment lengths, and damage parameters were compared for each sample across extraction treatments by using paired T tests or non-parametric Wilcoxon signed rank tests. For these analyses, samples were subdivided according to type of tissue. Normality assumptions were evaluated using a Shapiro-Wilk normality test (Table S3) and through visual examination of Quantile-Quantile plots and histograms of the difference between paired values as recommended by Ghasemi and Zahediasl (2012) (Figure S1 and S2). Correlations between variables were tested using Pearson's *r* as implemented in the R cor.test function.

Computational resources and R packages

This research was conducted using resources from the ASU High Performance Computing Saguaro environment. All calculations were performed in R 3.2.4. Scripts written for this project are available at: https://github.com/mnievesc/aDNAExtMethodsPaper_scripts. All plots and figures were generated using the ggplot2 (H Wickham, 2009), gridExtra (Auguie, 2016), tidyr (H Wickham, 2016) and reshape 2 (Hadley Wickham, 2007) packages or with R base graphics (R Core Team, 2016).

RESULTS

DNA yields

DNA yields were evaluated through flourometric quantifications of raw extracts (ng/µL) (Figure S3). These analyses did not reveal significant differences in mean DNA yields between samples extracted with either method irrespective of tissue (Table 2).

[Table 2 here]

Endogenous content and library complexity

For each DNA library, between 1 and 27 million reads were obtained after shotgun sequencing. After randomly selecting the same number of reads for each individual pair, between 1 and 9 million reads were analyzed per sample-treatment combination. Samples extracted with Method H tended to have a higher number of mapped, unique shotgun reads, but this difference was not statistically significant for either tissue (Figure 1). Percentages of endogenous content was calculated as the proportion of unique reads mapping to the reference (after duplicate removal and quality filtering) over the total amount of down sampled reads (Table S2). Most samples held <2% endogenous content, except for the chimpanzee sample, GB-7, which yielded >10% endogenous content, an up to sixteen-fold higher content than that found in the human libraries (Figure 2). This difference may be attributable to the younger age of the historic chimpanzee sample. Mean endogenous contents were not found to be significantly different for the samples extracted with either method in both tissue types (Figure S4A).

[Figures 1 and 2 here]

The relationship between clonality and endogenous content in the shotgun libraries is shown in Figure S5A. Sequence clonality (measured as fraction of duplicate sequence reads over total downsampled reads) is very low for all samples: <0.1%. However, the relationship between the two variables was not linear or significant for either tissue type (Teeth: Pearson's r = 0.4372, t = 2.062, df = 18, p=0.0538; Petrous portions: Pearson's r = 0.4419, t = 1.2068, df = 6, p=0.2729). To examine this question further and test which method produced higher complexity libraries, we used the *c_curve* function in preseq to estimate the number of distinct reads recovered for each library. High complexity libraries have a large proportion of distinct reads that map to different parts of the reference genome. Therefore, more parts of the reference are covered with a single sequencing experiment. In contrast, low complexity libraries have a large proportion of distinct reads that map to the same sites and therefore may have a strong bias and high redundancy (Head et al., 2014). In this dataset, complexity was high regardless of extraction method. Method D libraries had a slightly higher mean proportion of distinct reads than Method H libraries, irrespective of tissue type, but this difference was not statistically significant (Figure S4B). The relationship between complexity and endogenous content in the tested libraries is shown in Figure S5B. No significant correlation was observed between the two values (Teeth: Pearson's r = 0.1752, t = 0.7550, df = 18, p=0.4600; Petrous portions: Pearson's r = 0.5621, t = 0.665, df = 6, p=0.1470).

We used the *lc_extrap* function within preseq to predict the expected yield for a larger sequencing effort with the same libraries. This extrapolation analysis is highly sensitive to the amount of sequence data generated, and can give false estimates with low amounts of reads

(Daley & Smith, 2013). Therefore, this analysis was only possible for the two samples with the highest number of reads: PI-67 and GB-7. Figure 3 demonstrates that, in both cases, libraries constructed with Method H extracts were predicted to yield a higher amount of complex DNA fragments with deeper sequencing amounting to up to 10 billion reads. However, given that saturation of the complexity curve is reached early in the estimation, less than 1 million reads would be necessary to sequence all unique fragments predicted to be present in these libraries.

[Figure 3 here]

DNA fragment lengths and GC content

All samples, irrespective of extraction method had average DNA fragment lengths <100 bp. This small size is consistent with expectations for degraded remains (Briggs et al., 2007; Dabney et al., 2013b; Meyer et al., 2014) and similar to sizes obtained in previous aDNA research with tropical samples (Kehlmaier et al., 2017; Schroeder et al., 2015). Samples extracted with Method D yielded smaller average DNA fragment sizes (Tooth: 58.5 bp and Petrous portion: 53.7 bp) than those extracted with Method H (Tooth: 78.4 and Petrous portion: 72.2 bp) (Figure 4). Overlaid plots showing the length distribution of sequence reads, both before and after mapping and filtering, demonstrate that Method H libraries had a higher proportion of larger fragments (Figure S6-S8). Although this pattern of higher average fragment lengths in Method H libraries is evident in boxplots for both tooth and petrous portion samples, this difference was only found to be statistically significant in teeth. We suspect this finding is influenced by low statistical power due to the smaller size of the petrous portion sample. No significant correlation was found between endogenous content and read length in either tissue type (Teeth: Pearson's r = 0.3476 t = 1.5732, df = 18, p=0.1331; Petrous portions: Pearson's r = 0.4007, t = 1.0714, df = 6, p=0.3252) (Figure S9A).

[Figure 4 here]

Method D libraries had higher GC content irrespective of tissue. This difference was only found to be statistically significant in teeth and not in petrous portion samples (Table 2). A scatterplot of average DNA fragment lengths versus average percent GC content clearly distinguishes between samples generated with each method for both tissues (Figure 5). A significant negative correlation was found between average DNA fragment length and GC content for all samples (Teeth: Pearson's r = -0.4939, t = -2.410, df = 18, p=0.0268; Petrous portions: Pearson's r = -0.8357, t = -3.7286, df = 6, p=0.0097). No significant correlation was observed between percent GC and endogenous content (Teeth: Pearson's r = -0.4258, t = -1.9968 df = 18, p=0.0611; Petrous portions: Pearson's r = -0.6734, t = -2.2316, df = 6, p=0.0671) (Figure S9B).

DNA damage

All samples had high probabilities of C to T and G to A misincorporations caused by DNA damage at the first and last position of each fragment (>0.50) (see Supplementary File 3 for damage plots). This is consistent with known damage patterns of authentic aDNA sequences (Briggs et al., 2007; Dabney et al., 2013b; Overballe-Petersen et al., 2012). Neither of the three DNA damage patterns examined (λ,$\delta_{D,}$ δ_S) differed significantly between samples extracted with either extraction method (Figure S10).

DISCUSSION

In this research, we explored the performance of two extraction protocols on poorly preserved tooth and petrous portion remains excavated from tropical contexts. Our experimental results suggest that both Method D and Method H successfully recovered degraded genetic material from tooth and petrous portion samples. No statistically significant difference was observed in raw DNA yields, number of mapped, unique shotgun reads, endogenous DNA content, library complexity or postmortem damage patterns in shotgun reads. The latter suggests that neither method is biased against recovery of degraded DNA fragments. This finding is consistent with results previously reported by Gamba et al. (2016), who found that ancient samples extracted with several silica-based extraction methods did not exhibit different postmortem damage patterns. Other studies have demonstrated that modifying digestion or pre-digestion wash steps also had negligible effects on DNA damage profiles (Boessenkool et al., 2016; Damgaard et al., 2015).

Significant differences between methods were observed in DNA fragment length and percent GC content. DNA fragments recovered with Method H were, on average, 19 base pairs longer than those recovered with Method D, irrespective of tissue. Likewise, average GC content differed by at least 4 percentage points in teeth and by up to 9 percentage points in petrous portions. These differences were visible in boxplots for all tissue types but were only significant for comparisons with tooth extracts. The small size of the petrous portion sample likely resulted in low statistical power to detect significant differences. Because of this, we refrain from extrapolating meaningful conclusions based on the petrous portion datasets and note that further research with more comprehensive samples of petrous portion tissue is needed to resolve this question. The following discussion focuses on statistically significant trends observed in tooth samples only.

Extraction Method D was designed for recovery of ultrashort DNA fragments (Dabney et al., 2013a). Given that Method H uses the same binding and purification steps implemented in Method D, short DNA fragment loss may have occurred during the pre-digestion EDTA wash or during the extended digestion and decalcification step. Warinner et al. (2014) did not observe reductions in raw DNA yields after pre-extraction washes of calculus samples with EDTA. Other studies conducted with tooth and bone tissue also found no significant differences in average DNA fragment lengths recovered after modifying extraction procedures with extended digestion steps or bleach-based decontamination washes (Damgaard et al., 2015; Gamba et al., 2016; Korlevic et al., 2015). But a more recent report by Glocke and Meyer (2017) found that EDTA interferes with recovery of short DNA fragments. In general, reports comparing aDNA extraction methods have suggested that digestion time may be a strong influence on recovery rates and characteristics of endogenous aDNA. For instance, Damgaard et al. (2015) observed diminished aDNA recovery with digestion steps longer than one hour. More recently, Boessenkool et al. (2016) found that mean aDNA fragment lengths were smaller and GC content was higher in bone extractions performed with short digestions.

Method H implements an initial wash of 0.5M EDTA solution followed by an extended two-part digestion step in which bone powder is first incubated for one hour in lysis buffer, and then further kept overnight at 37°C. It is possible that further optimization of the EDTA wash solution and of subsequent digestion conditions, such as temperature and incubation time, are needed to avoid loss of small DNA fragments. Future optimization efforts may also benefit from separate library preparation and sequencing of EDTA wash and pre-digestion fractions to identify where small DNA fragments are being lost in the extraction process.

We observed that average GC content was at least four percentage points higher in paired tooth samples extracted with Method D versus Method H. But all samples, irrespective of extraction method, showed a decrease in GC content with larger average fragment size. This finding is consistent with previous research which has shown that differential DNA preservation can cause compositional bias towards higher GC content in ancient genomes composed of short DNA fragments (Briggs et al., 2007; Glocke & Meyer, 2017; Krause et al., 2010; Schuenemann et al., 2011). Higher GC content has also been correlated with lower contamination due to reduced presence of exogenous DNA (Racimo et al., 2016). As GC content can be strongly affected by amplification enzymes used in the library preparation process (Aird et al., 2011; Dabney & Meyer, 2012; Seguin-Orlando et al., 2015), all samples in this study were amplified with the same conditions so we consider this unlikely to explain the observed differences in base composition between extraction treatments. At a first glance, these results suggest that since Method D likely allowed for higher recovery rates of GC-rich DNA, it may be better suited for ancient tropical samples. However, we did not identify a significant correlation between percent GC and endogenous content (Figure S9B). High GC content can also lead to low sequence coverage in aDNA due to difficulty with mapping and alignment (Krause et al., 2010; Schuenemann et al., 2011). Although this problem may be alleviated somewhat by deep sequencing and high read depths, increased recovery of GC-rich DNA, may not necessarily lead to better results when read depth and coverage is inherently low, such as in poorly preserved tropical samples.

Lastly, we did not identify significant differences in the percent of distinct reads obtained from Method D and Method H samples after shotgun sequencing. However, extrapolation of predicted library complexity with the two best preserved samples (PI 67 and GB 7) indicated that Method H libraries would yield more unique DNA fragments upon repeated sequencing experiments. Complexity analyses are highly sensitive to the amount of sequence data generated. Low amounts of reads can lead to false estimates due to uncertainty of the extrapolation (Daley & Smith, 2013). This is reflected in the large confidence intervals in Figure 3, which indicate there is uncertainty in determining the exact point at which saturation would be reached after repeated sequencing. But, even with this caveat, the large difference between the complexity curves for both methods demonstrates that deeper sequencing would likely be most useful with libraries constructed from Method H versus Method D extracts. This pattern may be due to the higher number of unique reads after quality filtering that were recovered with Method H in the two samples examined (Table S2).

Several recent studies have found that the combination of extraction methods geared towards ultrashort DNA fragment recovery with single-stranded library preparation substantially increase endogenous aDNA yields (Barlow et al., 2016; Glocke & Meyer, 2017). While we are cognizant of these recent advances, in this work we have focused on libraries built with the more commonly used double stranded library protocol (Meyer & Kircher, 2010). Except for the historic chimpanzee sample, all archaeological remains examined in this study contained very low levels of endogenous DNA (<2%). This suggests that enrichment approaches are essential for increasing informative sequence content with poorly preserved tropical samples (Carpenter et al., 2013; Schroeder et al., 2015). Ongoing research in our laboratory has found that aDNA samples from Puerto Rico contain sufficient endogenous DNA for effective enrichment of complete mitochondrial DNA genomes (Nieves-Colón et al. 2017). Thus, here we follow recommendations by Wales et al. (2015) and focus on double-stranded library protocols which are better suited for studies geared towards enrichment of multi-copy organellar DNA.

Additionally, previous reports have demonstrated that extremely short molecules obtained after single-stranded library preparation (Gansauge et al., 2017; Gansauge & Meyer, 2013; Glocke & Meyer, 2017), are often lost during target enrichment capture (Ávila-Arcos et al., 2015).

CONCLUSIONS

This study has demonstrated that Method D and Method H were similarly efficient in recovering endogenous DNA in archaeological and historic skeletal samples from tropical contexts. However, significant differences exist in the composition of the recovered sequence data. Although libraries from Method H yielded more unique sequence reads, Method D recovered smaller aDNA fragments with higher GC content. Because of the exacerbated aDNA degradation that takes place in the tropics, we expect most informative sequence content to come from small DNA fragments in ancient remains (Allentoft et al., 2012; Hofreiter et al., 2015). Therefore, our findings suggest that, until further optimization of new protocols can take place, Method D continues to be the optimal choice for maximizing aDNA recovery in tropical tooth samples from ancient or historic contexts.

We also note that the insights derived from this work are restricted to tooth samples only. The smaller sample sizes obtained for petrous portion samples did not allow for conclusive statements regarding each method's performance with this tissue type. Future efforts to develop methodologies tailored for tropical aDNA samples will benefit from increased sampling of suitable petrous portions and other tissues, such as dental calculus. A larger dataset shall further allow for finer sub settings of the data so that study results will control for differences between relatively well versus poorly preserved samples and/or for differences in site-specific aDNA preservation patterns.

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Table 1. Skeletal samples included in the present study.

Sample	**Site**	**Region, Country**	**Köppen-Geiger environment classification**	**Sample Age (A.D.)**	**Tissue**	**Species**
PC E24	Punta Candelero	Humacao, Puerto Rico	Tropical monsoon	400-600[1]	Tooth	*Homo sapiens*
PC 117	Punta Candelero	Humacao, Puerto Rico	Tropical monsoon	400-600[1]	Tooth	*Homo sapiens*
T 251	Tibes	Ponce, Puerto Rico	Tropical monsoon	616[2]	Tooth	*Homo sapiens*
PI 67	Paso del Indio	Vega Baja, Puerto Rico	Tropical monsoon	1022[2]	Tooth	*Homo sapiens*
PI 388	Paso del Indio	Vega Baja, Puerto Rico	Tropical monsoon	822[2]	Tooth	*Homo sapiens*
AD 368	Yaxuna	Yucatán, México	Tropical savanna	6th century[1]	Tooth	*Homo sapiens*
AD 372	Yaxuna	Yucatán, México	Tropical savanna	6th century[1]	Petrous portion & Tooth	*Homo sapiens*
AD 373	Yaxuna	Yucatán, México	Tropical savanna	6th century[1]	Petrous portion & Tooth	*Homo sapiens*
AD 375	Yaxuna	Yucatán, México	Tropical savanna	6th century[1]	Petrous portion	*Homo sapiens*
AD 376	Yaxuna	Yucatán, México	Tropical savanna	6th century[1]	Petrous portion	*Homo sapiens*
AD 377	Yaxuna	Yucatán, México	Tropical savanna	6th century[1]	Tooth	*Homo sapiens*
GB 7	Gombe National Park	Kigome, Tanzania	Tropical savanna	1966[3]	Tooth	*Pan troglodytes schweinfurthii*

[1] Approximate date, based on archaeological context.
[2] Radiocarbon date median probability calAD.
[3] Date of individual death.

Table 2. Results of paired tests. Significant values P<0.05 bolded.

Test comparison	**Tissue**	**Paired test**	**Test statistic**	**DF**	**P-value**
DNA yields (ng/µL)	Tooth	T-test	t = -0.1924	9	0.8517
	Petrous portion	T-test	t = -1.2219	3	0.3090
Number of mapped, unique reads	Tooth	Wilcoxon signed ranks	V = 11	n/a	0.1054
	Petrous portion	Wilcoxon signed ranks	V = 0	n/a	0.1250
Percent endogenous content	Tooth	Wilcoxon signed ranks	V = 14	n/a	0.1849
	Petrous portion	Wilcoxon signed ranks	V = 0	n/a	0.125
Percent distinct reads	Tooth	Wilcoxon signed ranks	V = 41.5	n/a	0.1685
	Petrous portion	T-test	t = 1.1460	3	0.3348
Average fragment length	Tooth	Wilcoxon signed ranks	V = 0	n/a	**0.0019**
	Petrous portion	Wilcoxon signed ranks	V = 1	n/a	0.2500
Percent average GC content	Tooth	Wilcoxon signed ranks	V = 55	n/a	**0.0019**
	Petrous portion	Wilcoxon signed ranks	V = 10	n/a	0.1250
Damage parameter δ_D	Tooth	Wilcoxon signed ranks	V = 4	n/a	0.2324
	Petrous portion	Wilcoxon signed ranks	V = 2	n/a	0.3750
Damage parameter δ_S	Tooth	Wilcoxon signed ranks	V = 27	n/a	1.0000
	Petrous portion	Wilcoxon signed ranks	V = 5	n/a	1.0000
Damage parameter λ	Tooth	Wilcoxon signed ranks	V = 44	n/a	0.1005
	Petrous portion	Wilcoxon signed ranks	V - 8	n/a	0.3750

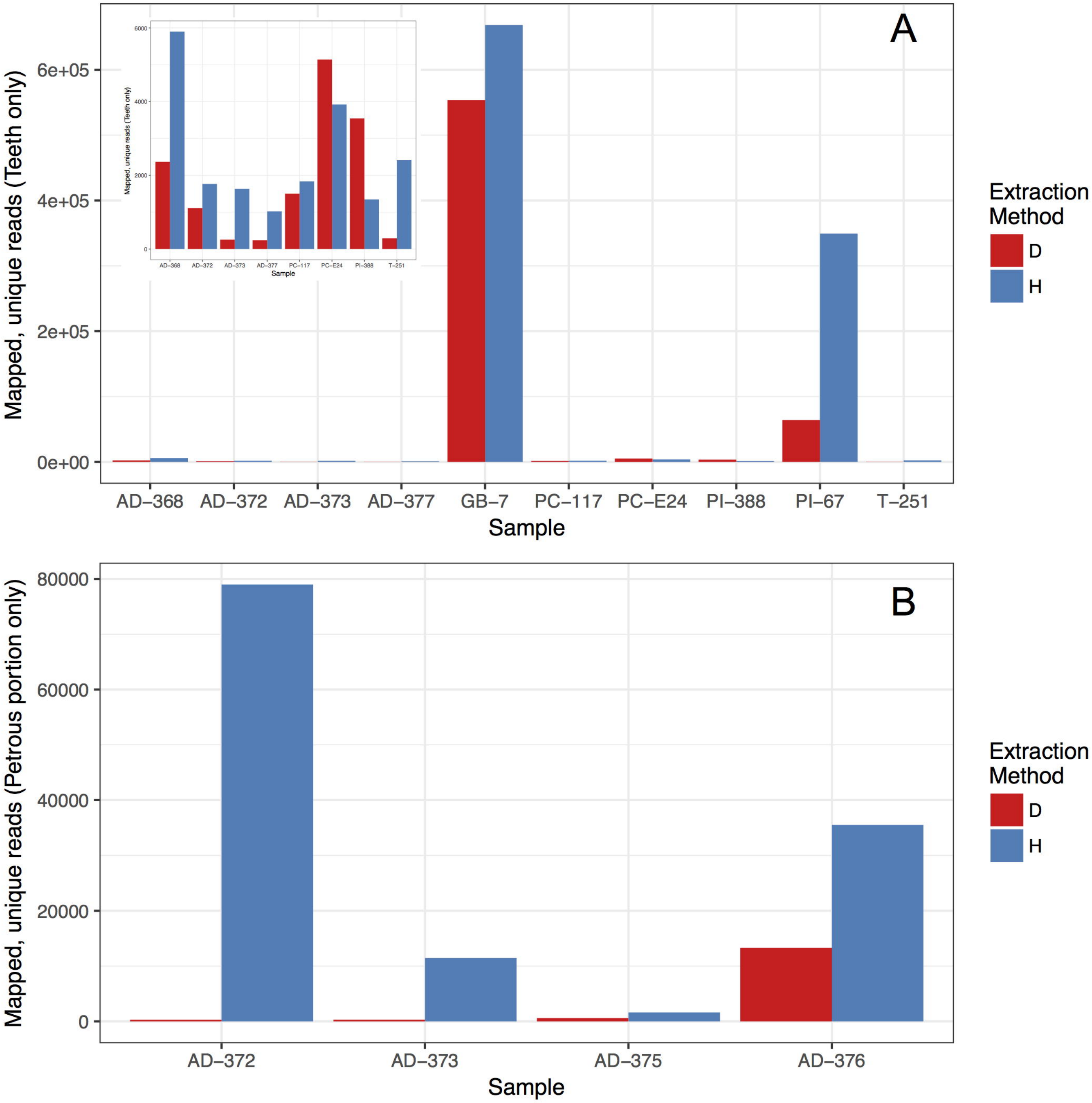
Figure 1. Number of mapped, unique reads per sample. (A) Tooth samples, inset zooms in to samples with less than 60,000 reads. (B) Petrous portion samples.

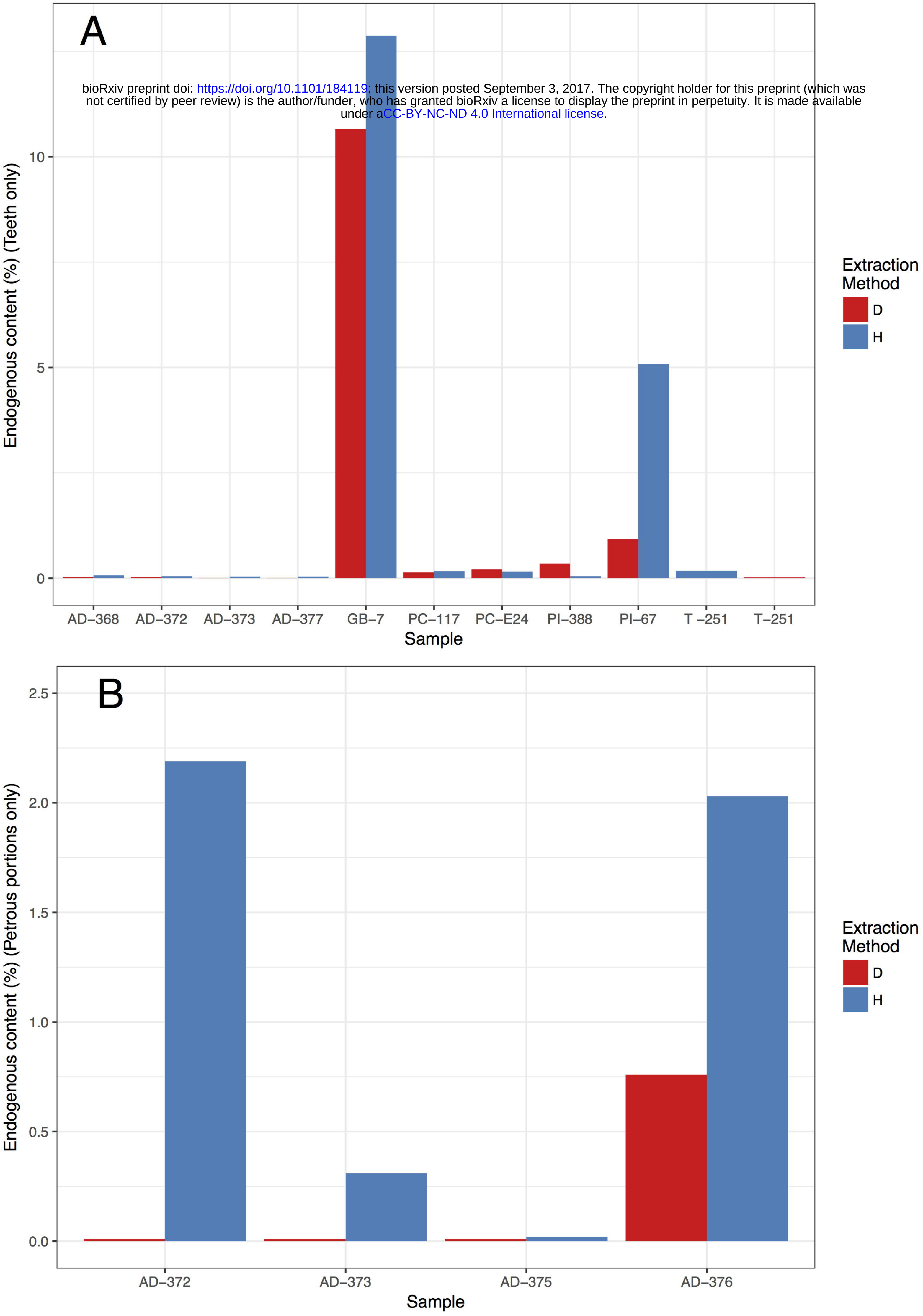
Figure 2. Per sample endogenous content in shotgun libraries. Percent endogenous content calculated as number of mapped, unique reads divided over total down sampled reads. (A) Tooth samples and (B) Petrous portion samples.

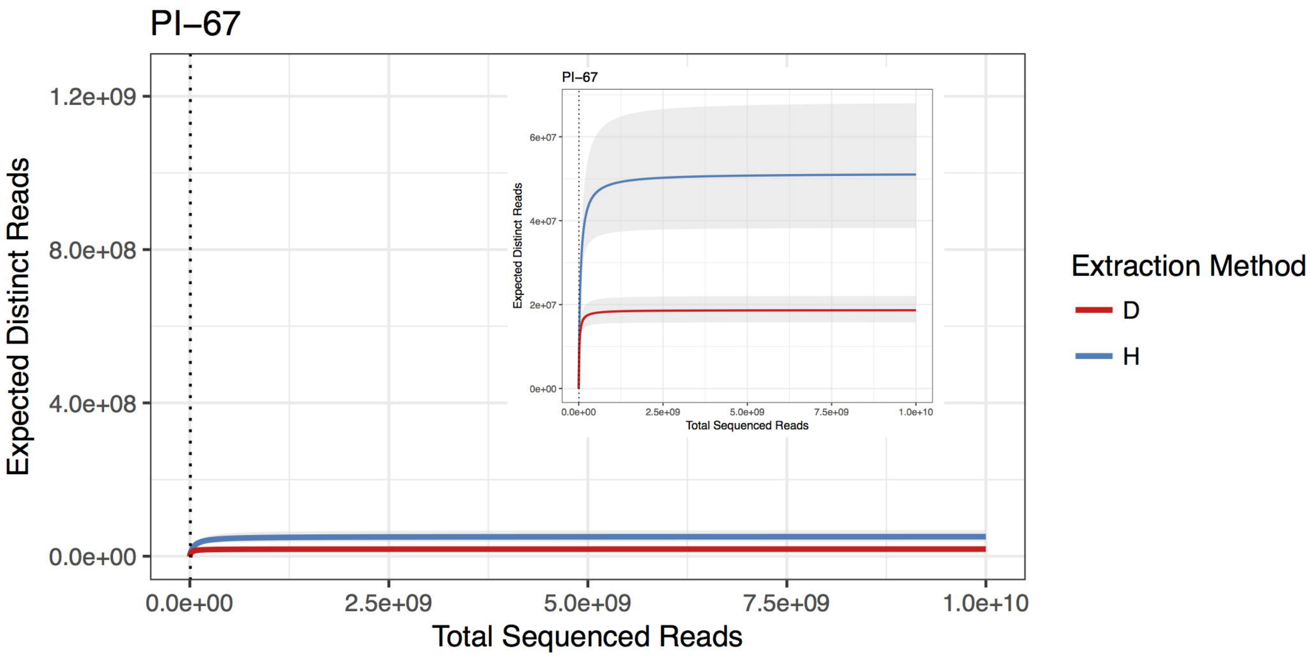
Figure 3. Extrapolation curves for shotgun library complexity estimation. Curves are shown for the two samples with highest number of reads: PI-67 and GB-7. Top inset shows zoomed in results for PI-67. Extrapolation curve and confidence interval estimation was performed in preseq using a step size of 100,000 and default sequencing effort of 10 billion reads. The dotted line denotes the number of reads randomly downsampled for each sample pair: 5.1 million reads for GB-7 and 6.8 million reads for PI-67.

Figure 4. Boxplots comparing distributions of DNA fragment lengths for tooth and petrous portion samples.

Figure 5. Percent GC content in shotgun libraries. (A) Boxplots, comparing distributions of average %GC content for tooth and petrous portion samples. (B) Scatterplot of mean fragment length versus average %GC content.

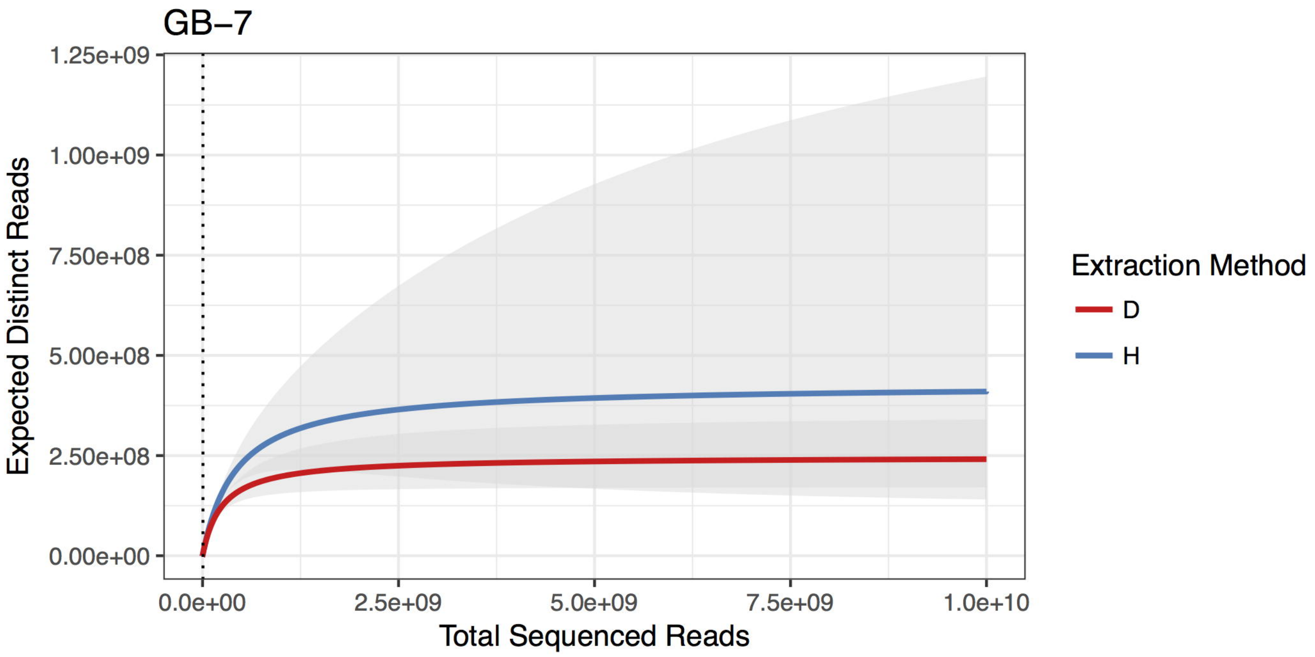
A
Mapped, unique reads (Teeth only)
6e+05
4e+05
2e+05
0e+00
AD−368
AD−372
AD−373
AD−377
GB−7
PC−117
PC−E24
PI−388
PI−67
T−251
Sample
Mapped, unique reads (Teeth only)
6000
4000
2000
0
AD−368
AD−372
AD−373
AD−377
PC−117
PC−E24
PI−388
T−251
Sample
Extraction Method
D
H
B
Mapped, unique reads (Petrous portion only)
80000
60000
40000
20000
0
AD−372
AD−373
AD−375
AD−376
Sample
Extraction Method
D
H


A
Endogenous content (%) (Teeth only)
10
5
0
AD–368
AD–372
AD–373
AD–377
GB–7
PC–117
PC–E24
PI–388
PI–67
T –251
T–251
Sample
Extraction Method
D
H
B
Endogenous content (%) (Petrous portions only)
2.5
2.0
1.5
1.0
0.5
0.0
AD–372
AD–373
AD–375
AD–376
Sample
Extraction Method
D
H



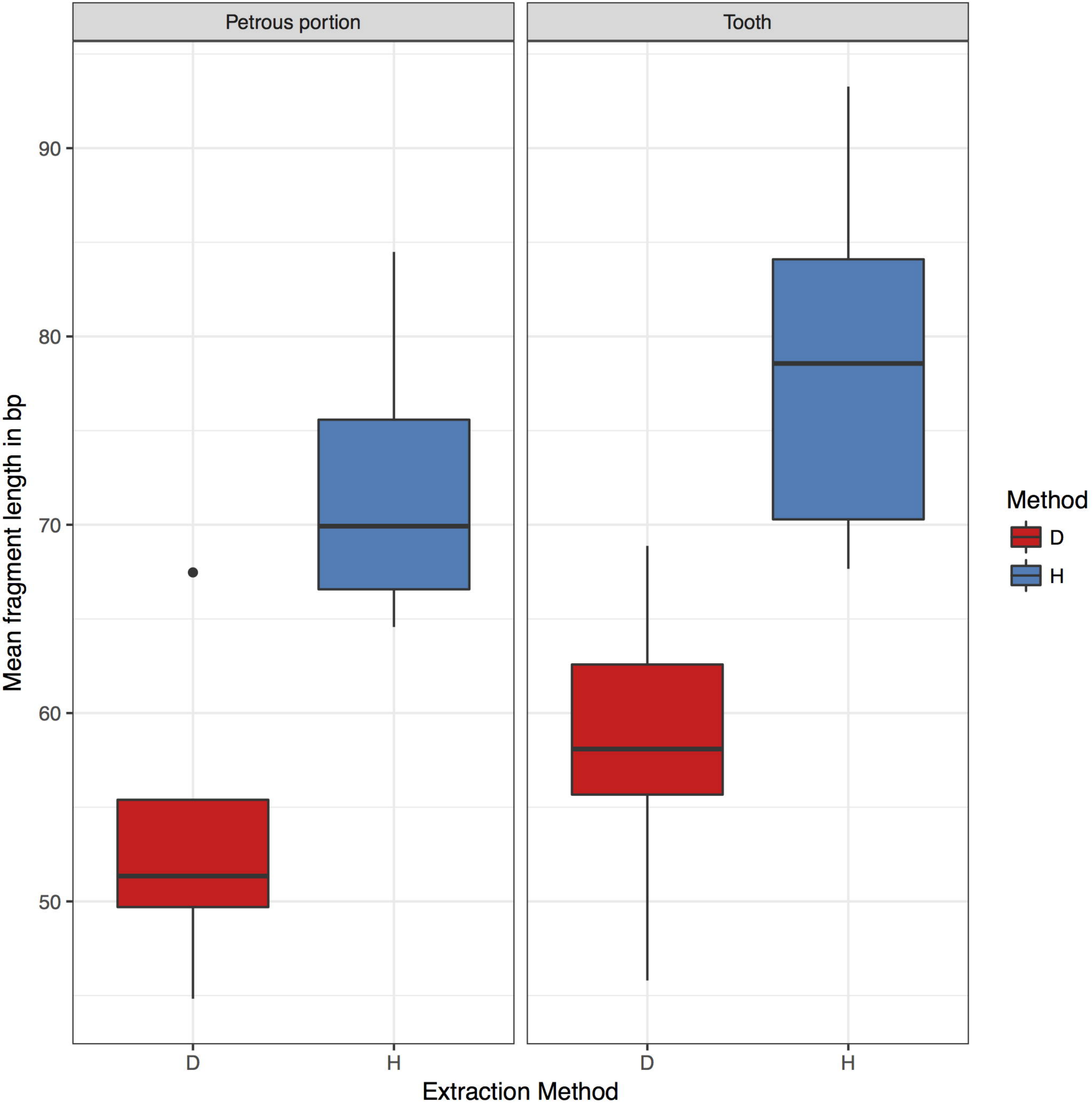
PI–67

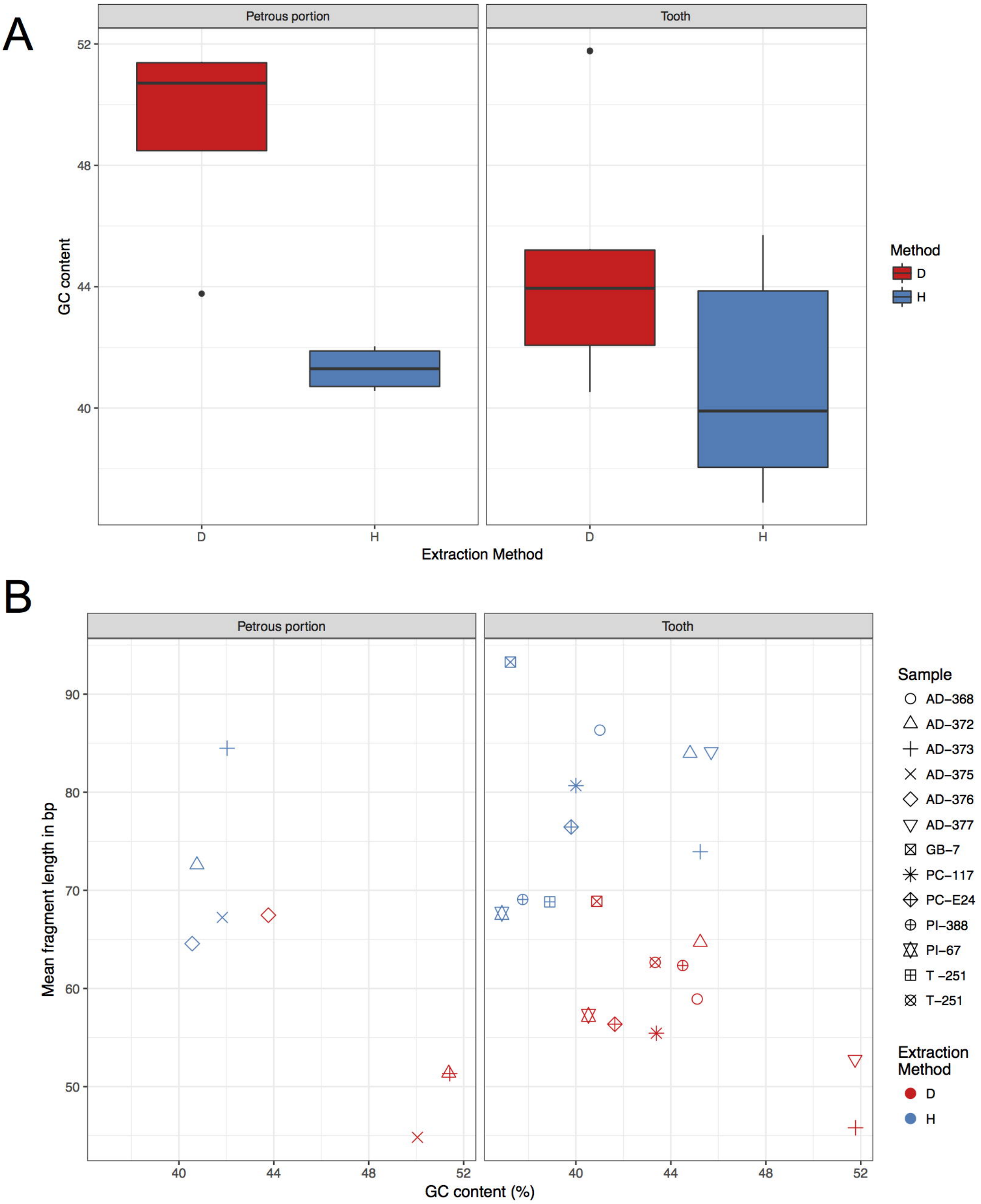
Expected Distinct Reads
1.2e+09
8.0e+08
4.0e+08
0.0e+00
0.0e+00
2.5e+09
5.0e+09
7.5e+09
1.0e+10
Total Sequenced Reads
PI–67
Expected Distinct Reads
6e+07
4e+07
2e+07
0e+00
0.0e+00
2.5e+09
5.0e+09
7.5e+09
1.0e+10
Total Sequenced Reads
Extraction Method
D
H



GB–7

Expected Distinct Reads
1.25e+09
1.00e+09
7.50e+08
5.00e+08
2.50e+08
0.00e+00
0.0e+00
2.5e+09
5.0e+09
7.5e+09
1.0e+10
Total Sequenced Reads
Extraction Method
D
H


Petrous portion
Tooth
90
80
70
60
50
Mean fragment length in bp
D
H
D
H
Extraction Method
Method
D
H


A
Petrous portion
Tooth
52
48
44
40
GC content
D
H
D
H
Extraction Method
Method
D
H
B
Petrous portion
Tooth
90
80
70
60
50
Mean fragment length in bp
40
44
48
52
40
44
48
52
GC content (%)
Sample
AD–368
AD–372
AD–373
AD–375
AD–376
AD–377
GB–7
PC–117
PC–E24
PI–388
PI–67
T –251
T–251
Extraction Method
D
H