Density separation of petrous bone powders for optimised ancient DNA yields

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Abstract

Density separation is a process routinely used to segregate minerals, organic matter, and even microplastics, from soils and sediments. Here we apply density separation to archaeological bone powders prior to DNA extraction to increase endogenous DNA recovery relative to a standard control extraction of the same powders. Using non-toxic heavy liquid solutions we separated powders from the petrous bones of 10 individuals of similar archaeological preservation into 8 density intervals (2.15 to 2.45 g/cm^3, in 0.05 increments). We found that the 2.30-2.35 and 2.35-2.40 g/cm^3 intervals yielded up to 5.28-fold more endogenous unique DNA than the corresponding standard extraction (and up to 8.53-fold before duplicate read removal), while maintaining signals of ancient DNA authenticity and not reducing library complexity. Although small 0.05 g/cm^3 intervals may maximally optimise yields, a single separation to remove materials with a density above 2.40 g/cm^3 yielded up to 2.57-fold more endogenous DNA on average, which enables the simultaneous separation of samples that vary in preservation or in the type of material analysed. While requiring no new ancient DNA lab equipment and fewer than 30 minutes of extra lab work, the implementation of density separation prior to DNA extraction can substantially boost endogenous DNA yields without decreasing library complexity. Although subsequent studies are required, we present theoretical and practical foundations that may prove useful when applied to other ancient DNA substrates such as teeth, other bones, and sediments.

Introduction

Over the past decade there has been a concerted effort to improve the efficiency of DNA recovery from irreplaceable archaeological specimens, such as human bones and teeth. The majority of ancient DNA research is now carried out using skeletal elements that have biologically higher endogenous DNA contents, such as petrous bones, ear ossicles, and tooth cementum (Gamba et al. 2014; Pinhasi et al. 2019, 2015; Harney et al. 2021; Hansen et al. 2017; Damgaard et al. 2015; Sirak

et al. 2020). Ancient DNA-specific wet lab protocols have increased the quantity of DNA isolated and extracted (Dabney et al. 2013; Rohland et al. 2018), and improved the efficiency of DNA library construction using single-stranded molecules (Kapp et al. 2021; Gansauge et al. 2020; Gansauge and Meyer 2019, 2013). 'Pre-treatment' steps, such as cleaning samples with a weak sodium hypochlorite (bleach) solution (Kemp and Smith 2005), implementing a chemical or enzymatic 'pre-digestion' step (Damgaard et al. 2015; Korlević et al. 2015; Schroeder et al. 2019), or a combination of the two (Boessenkool et al. 2017), aim to reduce contamination and maximise endogenous DNA yields; however, these steps reduce the complexity of genomic sequencing libraries, negatively influencing downstream analyses. Here, we present a different type of 'pre-treatment' step that improves endogenous DNA yields, while not decreasing the complexity of sequencing libraries.

After the death of a vertebrate organism, the chemical composition of its skeletal remains immediately starts to be altered by diagenetic processes such as hydrolysis, enzymatic action, mineral dissolution, and microbial colonisation (Hedges 2002; Rasmussen et al. 2019; Kendall et al. 2018; Bell et al. 2001; Booth 2016). These processes have substantial and irreversible effects on the structure and composition of bone, inducing collagen loss and alteration of the ratio of organic to inorganic fractions. Microorganisms, in particular, have been shown to play an important role in the mineralisation of bone elements, and can, therefore, cause deviations in localised density (Bell et al. 2001; Daniel and Chin 2010). As this process is one of the main pathways for the introduction of exogenous contaminant DNA, avoiding or eliminating these exogenous mineralised pockets prior to DNA extraction may facilitate the recovery of greater amounts of endogenous DNA.

In vivo bone naturally contains regions of differing densities. As an individual ages, new bone formation is accompanied by the progressive mineralisation of existing bone matrix and osteocytes, in parallel with bone remodelling generating new osteonal systems, leading to regions of different densities within the same bone (Bell et al. 2001, 2008; Kendall et al. 2018). In petrous bones and ear ossicles, however, there is a lack of bone remodelling after approximately 24 weeks *in utero*

(Hernandez et al. 2004), contributing to a high concentration of mineralised osteocytes in relation to other bones (Hernandez et al. 2004; Bell et al. 2008; Ibrahim et al. 2022), and this has been hypothesised to be one of the main determinants of the success of petrous bones in the retrieval of ancient DNA (Pinhasi et al. 2019; Ibrahim et al. 2022).

Previous work has used density separation, also referred to as fractionation, to isolate elements of different densities before applying isotopic analysis to successfully reconstruct the dietary habits of archaeological individuals over the last 15 years of their lives (Bell et al. 2001). However, to the best of our knowledge, density separation has not yet been applied to the process of recovering ancient DNA from archaeological bone powders. In theory, it could be used to not only separate endogenous bone elements of different densities, specifically including mineralised osteocytes (like in the isotopic study just mentioned) but also to separate non-endogenous clusters of mineralised microorganisms and environmental sediments that sometimes cannot be removed entirely during sample processing in an ancient DNA lab.

Here we present a new method for isolating the most endogenous DNA-rich fractions of petrous bone powder without additional ancient DNA lab equipment and using a non-toxic heavy liquid named sodium polytungstate (SPT). The main objective of this work was to identify density interval(s) that contained more endogenous DNA when compared to a standard extraction of bone powder from the same individual, establishing the validity of using density separation as a technique to improve DNA recovery.

To reduce the number of external and differentiating variables that could influence our results, we restricted our analyses to petrous bones from two temperate locations in Europe (more details on the process of sample selection are described in the **Methods** section). According to the "Allen Ancient DNA Resource", of the 8797 libraries with a clearly stated skeletal source of DNA, the petrous was used for analysis 4839 times, representing more than half of all cases (teeth were used 3161 times, representing approximately another one-third of all cases, and all other elements were

used in only ~10% of cases), and is therefore the most likely element to which such new methodology would be applied to (https://reich.hms.harvard.edu/allen-ancient-dna-resource-aadr-downloadable-genotypes-present-day-and-ancient-dna-data, version 54.1.p1).

Results

As there is no published data or protocols for the recovery of ancient DNA from archaeological bone powder following a density separation step, we began by following a protocol similar that presented in Bell et al. (2001), running a small preliminary experiment (more details in the **Methods** section) to achieve the protocol presented here (illustrated in **Figure 1**). Any use of the terms "density" and "density intervals" in what follows refers to a measure that approximates true densities, instead of bulk densities, as the latter are calculated considering bone porosity as contributing to the units of space and have, therefore, substantially lower values than the true densities (Lee Lyman 2021).

We selected 10 petrous bones from remains excavated from two archaeological locations in temperate parts of Europe, namely the Bronze/Iron Age sites of Praha 5 - Malà Ohrada/Jinonice, respectively, in the Czech Republic (n=4) and the Late Antiquity/Early Medieval necropolis of Castel Sozzio (Civitella D'Agliano, Viterbo), in Italy (n=6) (**Supplementary Table S1**). We separated from macroscopically homogeneous bone powder generated for each individual 150 mg of powder for sequential separation over 8 density intervals that were identified in our preliminary experiment as the most relevant (<2.15, 2.15-2.20, 2.20-2.25, 2.25-2.30, 2.30-2.35, 2.35-2.40, 2.40-2.45, >2.45 g/cm^3). For convenience, all intervals are from here on referred to by their highest value (e.g. interval 2.20 refers to the interval range 2.15-2.20, and 2.40 to the range 2.35-2.40). We separated a further 50 mg of powder from the same homogeneous powder for standard ancient DNA extraction that followed a previously published protocol (Dabney et al. 2013) and did not include any density separation step. After sequential separation and suspension of the powder particles over the 8 density intervals, each tube's liquid density was lowered and washed with Tris-EDTA (TE) buffer to

repellet the suspended bone powder and remove all traces of SPT (**Figure 1**, details in **Methods** section).

Subsequently, DNA extraction took place using the same protocol as for the standard extractions (**Figure 1**). All 90 libraries (10×8 intervals plus 10 standard extractions) were screened using low-coverage shotgun sequencing ("Sequencing Group 1"), and the resulting sequencing data and quality metrics were assessed (**Supplementary Table S1**). After identifying the 2 best density intervals from "Sequencing Group 1" by their percentages of unique endogenous reads, we carried out additional shotgun sequencing of the same libraries (also further sequencing the standard extraction libraries) in order to obtain greater amounts of data to allow for more robust comparisons ("Sequencing Group 2"). The data from the different sequencing runs was then merged (**Supplementary Table S1**) and, to ensure comparability and absence of bias due to unequal sequencing yields, was randomly subsampled to equal numbers of reads within each individual, using *seqtk* (github.com/lh3/seqtk) (**Supplementary Table S2**). To statistically compare the data from the standard extractions and the best density intervals, we computed paired Wilcoxon signed-rank tests with a *P*-value threshold of significance of 0.05, and a minimum of 6 paired observations, and using the normalised, subsampled, and merged data for all metrics except for contamination estimates, as the latter should not be affected by random subsampling and will benefit from more data for increased accuracy (**Supplementary Table S1** for contamination and **Supplementary Table S3** for all other metrics).

Overall, the application of the density separation protocol was successful for all individuals and densities, confirming that using SPT does not exert a negative influence on the DNA molecules or the process of DNA extraction itself. We did not weigh the exact powder amounts separated in each interval to avoid the potential introduction of contamination during drying steps (the weight would have also been inaccurate because the powder was wet). However, a generalised sequential increase in separated repelleted powder quantities was observed along with the density, from nearly

negligible amounts at <2.15 to substantial amounts at 2.40 and 2.45, with the largest pellet always present in the heaviest density interval, >2.45 g/cm^3 (**Supplementary Figure S1, Supplementary Figure S2**). The assessment of the screening data across the 8 density intervals, indicates that the relative amounts of unique endogenous DNA (measured by the ratio of non-duplicated reads aligned to the human genome by the total number of reads) increased from 2.15 to 2.40 g/cm^3, with the highest endogenous DNA contents always present in the higher intervals - either at 2.35 or 2.40 (**Figure 2**, **Supplementary Table S1**). We note a single exception for individual P9884, for whom the optimal interval was 2.45 g/cm^3 (**Supplementary Table S1)**. After re-sequencing and normalising the data, these best intervals and standard extractions were compared further (**Supplementary Table S2**). Here, the average fraction of endogenous unique DNA reads for the standard extractions was 13.25% (range 4.79-26.55%), whereas for the best SPT intervals it was 32.86% (range 20.76-45.01%), represented by an average improvement of 3.04-fold (range 1.53-5.28) per individual (**Figure 3A**, **Supplementary Table S2**). The smallest change was for individual P9898, from 26.55% endogenous DNA in the standard extraction powder, to 40.62% in the 2.40 g/cm^3 interval, whereas the largest was for individual 2338, from 5.41% in the standard extraction, to 28.54% in the 2.35 g/cm^3 interval (**Figure 3A, Supplementary Table S2)**. Consequently, the endogenous contents of the standard extractions were found to be inversely correlated to the fold-increase obtained in the best SPT intervals (**Figure 4**).

These paired differences in endogenous DNA yields were statistically significant (*P*-value = 0.001953), and were the result of an average increase of 3.04-fold in the number of unique aligned reads (*P*-value = 0.001953) (**Figure 3A, Supplementary Table S2, Supplementary Table S3**). However, these changes can potentially be higher in samples with lower library saturation and fraction of duplicated reads after alignment, as we were able to achieve an 8.53-fold increase in endogenous contents for individual 2338 before the removal of duplicated reads, from 6.24% to 53.20% (**Supplementary Table S2**).

We estimated individual library contamination using a recently developed method that requires only 0.02× whole genome coverage per sample (Huang and Ringbauer 2021). This method models and quantifies mismatches in haploid X Chromosomes as contamination, and is therefore restricted to individuals who are molecularly sexed as male, which in our case corresponded to 8 out of 10 individuals. The average contamination for all but one library above the 0.02× threshold in "Sequencing Group 1" (n=32) was estimated to be 2.87% (range 1.37-4.48%), which stands below the typical threshold of 5% for ancient DNA (Fu et al. 2013; Nakatsuka et al. 2020) (Supplementary Table S1). One outlier had an estimated 9.9% contamination on the lower density interval (<2.15 g/cm3 for individual P9898), which is likely an isolated observation, as the average contamination for the same individual's other 6 intervals and standard extraction was 2.57% (Supplementary Table S1). By then looking at the merged higher coverage data for the best intervals and the standard extraction ("Sequencing Group 2", non-subsampled in order to have increased analytical power), the average contamination among all was estimated to be 2.65% (range 1.30-4.80%), with a non-significant difference between the best SPT intervals (average 2.76%) and the standard extractions (average 2.55%) (P-value = 0.5469) (**Figure 3B, Supplementary Table S1, Supplementary Table S3**). The negative controls included in all stages of the laboratory work support zero to negligible ancient cross-contamination, as 12/13 libraries had no terminal deamination, and the average duplication rate after polymerase chain reaction (PCR) amplification to plateau was high, at 64% (Supplementary Table S1). One control did have 0.02 terminal deamination, but only 302 sequences remained after filtering with PMDtools (threshold 3) to isolate those with the highest likelihood of being ancient (Skoglund et al. 2014).

We then investigated if separation using a wider density interval would still give better results than a standard extraction without a density separation step. A wider range that still improves DNA recovery with no introduction of contamination would enable this method to be applied simultaneously to multiple and varied samples with different preservation, taphonomic histories, and maybe even to different skeletal or other bioarchaeological DNA substrates, increasing the

overall applicability of this technique. Using the screening data from "Sequencing Group 1", we found that the average endogenous DNA yields for the combined intervals 2.25, 2.30, 2.35, and 2.40 were still statistically higher than the yields of the standard extraction (P-value = 0.003906), with an average increase of 2.35-fold (range 0.81-5.24) (**Supplementary Table S1, Supplementary Table S3**). To investigate this further, we used the remaining powder (between 55 and 124 mg) from the exact same batches of 7 individuals after having measured the initial 150 + 50 mg, and separated them at a single density of 2.40 g/cm^3 to formally test the possibility of obtaining improved yields with a single practical separation. The average endogenous contents for the <2.40 intervals was 35.05% (range 25.75-47.61%), corresponding to an increase of 2.83-fold compared to the standard extractions (P-value = 0.01563) (**Supplementary Table S3, Supplementary Table S4**). Furthermore, for every individual, the single <2.40 interval yield was higher than the corresponding standard extraction, independently of the initial powder amount. This suggests that, while this wider density interval performed slightly worse than the narrower ones, a single separation at 2.40 g/cm^3 still provides an improvement in endogenous yields relative to a standard extraction without a preceding density separation step, and that smaller initial powder amounts down to at least 50 mg can likely be used (**Supplementary Table S3, Supplementary Table S4**).

In regards to the effects of the overall increase in endogenous contents of the main set of 10 samples in other quantitative metrics, such as nuclear genomic and mitochondrial coverages, we observed an increase in both of these metrics for the best SPT intervals was statistically significant (P-value = 0.001953, for both), with the average nuclear coverage increasing on average 2.94-fold (range 1.58-5.04), and the mitochondrial coverage on average 3.12-fold (range 1.87-5.01) (**Supplementary Table S2, Supplementary Table S3**). Similarly, when we looked at the curves produced by *preseq*'s (Daley and Smith 2013) *lc_extrap* command as a measure of library complexity, which extrapolates the expected number of distinct reads after extensive sequencing from duplication rates, we found a statistically significant average increase of 1.77-fold in the number of distinct reads at a sequencing effort of 100 million reads for the best SPT intervals (P-

value = 0.001953) (**Figure 3C, Figure 5**, **Supplementary Table S3**, **Supplementary Table S5**). Although these results can be interpreted as suggesting an increase in observed library complexity, we were not able to investigate changes in complexity per milligram of input powder due to lack of the latter measurements.

Lastly, the average read length of 57 base pairs (bp) in the standard extracts (range 50-67 bp) was not statistically different from the average of 55 bp in the best SPT intervals (range 51-63 bp) (P-value = 0.1641)(Sawyer et al. 2012; Green et al. 2009) (**Supplementary Table S2, Supplementary Table S3**). However, we observed significant differences in deamination frequencies in the terminal bases of the DNA. These chemical alterations to the DNA in the form of C>T and G>A changes have been shown to be characteristic of ancient DNA, and are one of the most important metrics for assessing authenticity (Sawyer et al. 2012; Green et al. 2009; Ginolhac et al. 2011). We observed an increase in the average deamination frequencies of terminal bases of the 5' end of the DNA sequences from 0.32 in the standard extracts (range 0.27-0.40) to 0.34 in the best SPT intervals (range 0.31-0.44), with a P-value of 0.003906 (**Supplementary Table S2**, **Supplementary Table S3**). A similar pattern was observed for the 3' end (**Supplementary Table S2**, **Supplementary Table S3**). Our data does not suggest that these were caused by chemical exposure to the SPT and/or its low pH, as the intervals with the longest exposure did not show higher deamination. Furthermore, an expected pattern where higher contamination would lead to lower deamination was also not observed. Nevertheless, future investigations will be able to shed more light into this situation.

Discussion

We carried out density separation of bone powder across 8 sequential density intervals as an alternative type of 'pre-treatment' step to increase unique endogenous ancient DNA yields without decreasing library complexity or negatively impacting DNA authenticity. When a density separation step was implemented, we observed an up to 5.28-fold increase in unique endogenous DNA recovery, reflecting a more efficient exclusion of contaminant-rich bone elements, leading to higher

ratios of endogenous DNA on normalised total read numbers. All authenticity metrics examined were similar to those for the standard extractions, and library complexity based on expected distinct reads extrapolations was never reduced for the best SPT intervals, contrary to the results observed following other pre-treatment techniques such as the use of bleach to reduce surface contamination. As such, a density separation step can provide an efficient way to improve the cost efficiency of ancient DNA sequencing without requiring additional lab equipment.

Notably, the largest improvements in endogenous unique DNA yield were observed for the samples with the lowest yields obtained for the standard extraction. On average, we saw an increase of 4.31-fold on samples with less than 10% endogenous DNA yield on the standard extraction, whereas for samples with over 10% that increase was 2.19-fold, suggesting that density separation may enable the re-evaluation and analysis of previously processed samples that failed to pass the quality and quantity thresholds that rendered them inappropriate for further sequencing or population genomics analysis.

The consistency in the best density intervals identified across all samples confirms that the theoretical background of this method is valid, and that, in samples with similar preservation, particles rich in endogenous DNA will be concentrated at a specific shared density range. It also confirms the validity of our selection of intervals for this experiment, as the best intervals included the higher density intervals (2.35 and 2.40), but not, except for a single sample, the interval of highest density (2.45). These results also match what is described in Bell *et al.* (2001) regarding the 2.30-2.40 g/cm^3 interval comprising interstitial bone and representing the most highly mineralised, and older, human bone elements, which is what is to be expected to be found in the cochlea due to its lack of bone remodelling and high concentration of mineralised osteocytes containing endogenous DNA (Busse et al. 2010; Bell et al. 2008). However, and considering that different bone densities can be associated with different mineralisation levels (Bell et al. 2008) variations between

the various elements of the skeleton are expected, and further work should focus on the identification of each element's most optimal density intervals.

For example, as most bones are expected to have an overall lower density than the cochlea due to constant bone remodelling which results in a higher proportion of recently-formed, lower density, bone, those intervals might be slightly lower, but nevertheless above 1.7 g/cm^3 (Lee Lyman 2021; Bell et al. 2001; Simmons et al. 1991; Cameron et al. 1993; Currey 1984). In the case of (fresh) teeth, specifically, it has been shown that cementum and dentin can be separated both from enamel and each other, using individual density fractionation intervals (2.04, 2.20-2.40, and 2.70-2.80 g/cm^3, respectively) (Brekhus and Armstrong 1935). On the other hand, poorly preserved and/or bones that have undergone substantial mineralisation and fossilisation over long time periods, may present an overall shift towards higher density intervals (Sillen 1981; Bell et al. 2001; Daniel and Chin 2010). Although no studies exist for human bones, this was demonstrated in a gazelle bone from the Natufian period where the general density range moved from 1.9-2.3 g/cm^3 to 2.4-2.6 g/cm^3 (Sillen 1981; Bell et al. 2001). Thus, further studies may help us to understand if such shifts happen differentially across human bones, the rates at which they occur, and provide additional insight into where within the bone tissue structure the DNA is preserved (if it is still present).

Moreover, in a similar manner as to how oxygen isotopes from tooth enamel can be used to investigate individual mobility between childhood and adulthood (Pellegrini et al. 2016; Budd et al. 2004) the fact that different mineralisation and density levels within the same bone are associated with differential bone formation over the last 15 years of life of an individual (Bell et al. 2008, 2001), may prove extremely useful for future studies involving changes in ancient epigenetics and methylation patterns over a substantial period of the individual's life. Furthermore, since our proposed protocol includes several SPT wash steps, we argue that the fractions not used for ancient DNA isolation could be used in parallel for isotopic analysis. However, experimental confirmation of lack of SPT reactivity is required.

Sedimentary samples may also be a good candidate substrate for the application of density separation for ancient DNA extraction, as it may provide a way to segregate bone elements from substantially heavier and lighter soil minerals that should be richer in environmental DNA. Similarly, the same is likely also true for petrous bone powder samples obtained through cranial base drilling of complete skulls, as these often include soil particles that become loose due to drilling vibrations, and are very hard to be completely excluded from the final powder (Sirak et al. 2017).

All these possibilities create an almost endless array of follow-up investigations to the application of density separation in the field of ancient DNA, and may even open up new possibilities for re-evaluating old stored powders or bones that previously did not yield workable amounts of endogenous DNA. By using a non-toxic, inexpensive, and easily accessible chemical reagent, and requiring only a microcentrifuge (which is a staple equipment in any molecular DNA lab), density separation using SPT can be integrated into any existing ancient DNA laboratorial pipeline as a pre-treatment step before DNA extraction that optimises DNA recovery.

Methods

Selection of density intervals through preliminary experimentation

By following existing research (Bell et al. 2001; Simmons et al. 1991), and to have a more general view of the distribution of ancient DNA during the first application of SPT density separation to archaeological bone powder, we initially separated 300 mg from 3 ancient individuals in 0.2 g/cm^3 intervals for the range of 1.6 to 2.8 g/cm^3. Due to an imperfect first adaptation of the method to ancient bone powders, most of the tested intervals were lost during processing and were not sequenced; however, we did obtain data that showed that the intervals below 2.00 and above 2.40 g/cm^3 yielded no endogenous DNA, or extremely small amounts. Instead, we found that most of the DNA was present between 2.20-2.40 g/cm^3. This preliminary data was in agreement with the results of the existing literature indicating that the best density intervals for ancient human bones was

between 2.00 and 2.40 g/cm^3, and more specifically 2.30-2.40 g/cm^3 for the more mineralised bone elements (Bell et al. 2001), and formed the basis for our future work. We therefore decided to analyse the 2.20-2.40 range, and to increase specificity and accuracy we used smaller 0.05 intervals. Given the observation that even minute amounts of bone powder were being separated, instead of the large initial amount of 300 mg of powder we subsequently used only 150 mg. Lastly, we also included one extra interval on each side, ending up with 2.15 and 2.45 g/cm^3, plus the analysis of the elements below 2.15 and above 2.45, resulting in 8 density intervals per sample, plus the standard extraction, in a total of 90 independent powder aliquots for DNA extraction and sequencing (<2.15, 2.20, 2.25, 2.30, 2.35, 2.40, 2,45, >2.45, Standard Extraction).

Sample selection and processing

Due to the varied chemical and physical composition of different skeletal elements, and how they are affected by many different variables such as overall preservation, taphonomic pathways, chronological age, exposure to sudden environmental changes, and others, measured densities will vary on an individual and elemental basis. A global and optimal density separation method for every single individual or sample type is therefore very unlikely. For this study, in order to reduce the number of external variables that could affect ancient DNA yields and quality, and therefore inhibit our ability to detect statistically significant patterns and trends, we decided to use 2 sets of skeletal elements from individuals with similar preservation. The petrous bones were processed according to published protocols (Pinhasi et al. 2019) in dedicated ancient DNA facilities at the Department of Evolutionary Anthropology of the University of Vienna, where the cochleae were isolated and milled to homogeneous powder in a Retsch Ball Mixer Mill MM 400 at maximum frequency (30 Hz) for two 30 second sessions with a 10 second pause between to allow for the dissipation of any heat built up. Two powder aliquots were prepared for each individual: one of 150mg for density separation, and another of 50mg for ancient DNA extraction using traditional protocols.

Density separation and DNA extractions

SPT (TC-Tungsten Compounds GmbH) was chosen to create heavy liquid solutions due to its ease and safety of use for the researcher, being inorganic, non-toxic, and highly soluble in water. Furthermore, it has a large density range (~1.10 to 3.1 g/cm^3), and is thought to be inert against DNA. On the other hand, changes in its pH (2-3) affect its stability and may lead to chemical modifications and the transformation into sodium paratungstate, which is insoluble in water, and when in contact with ethanol, SPT creates a white precipitate that may have an unknown effect on, or inhibit, PCR reactions (R Kamps 2017, pers. comm.). DNA library inhibition was not assessed during our experiments as this white precipitate was never observed. To ensure precision down to the 0.05 g/cm^3 intervals, we started by preparing SPT solutions for the 8 specified densities by creating a calibration curve based on the 3-degree polynomial trend line of 15 measured densities (**Supplementary Table S6**). Before use, these SPT solutions were then UVed for 10 minutes. Starting with the lowest density solution (2.15), 1 mL of it was transferred to a 2mL Eppendorf tube containing 150mg of bone powder, and the contents were thoroughly mixed by vortexing. After 4 minutes of centrifugation at maximum speed (20,238 g), all powder elements heavier than the density used (here 2.15 g/cm^3) sank into a pellet, while the supernatant contained a suspension of elements lighter than or equal to 2.15 g/cm^3. The supernatant was transferred into a new 2mL tube, and labelled according to its density interval, here <2.15 (**Figure 1**). Although not specifically required, wide bore tips can be useful for this step. Then, 1mL of the next density, 2.20, was transferred into the tube with the pellet, and the process was repeated. In this case, elements lighter than 2.20 (and heavier than the previous density, 2.15) remained in suspension, while the heavier elements, again, formed the pellet that was used for the next density separation. This process was then repeated for all densities. The last tube obtained using the SPT solution of 2.45 g/cm3 contained a pellet with elements heavier than 2.45, and a supernatant with elements between 2.40 and 2.45. As mentioned above, both were further processed, extracted, and sequenced.

After all separations were achieved, each tube with the newly separated elements suspended in 1mL of SPT went through a wash and repelleting phase, by adding 1mL of TE buffer to each tube in order to lower the liquid solution's density, and force the pelleting of the now-heavier bone elements. The addition of 1mL of TE buffer lowers the density of the SPT solutions to between 1.67 and 1.73 g/cm^3, for the 2.15 and the 2.40 tubes, respectively, which should be lower than any existing bone elements and, therefore, force them to repellet. After vortexing, each tube was centrifuged at maximum speed for 4 minutes, and the supernatant containing low density SPT+TE discarded. In order to ensure a thorough removal of the SPT, this wash/repelleting step was repeated 2 times. Although not requiring repelleting, the >2.45 tube with the final pellet was also washed 3 times to remove all SPT traces. If this, or any other pellet, is somewhat large (e.g. >50-100mg) using 2mL of TE buffer per wash is suggested.

After the washes, all pellets were ready for standard DNA extraction, which was performed using the Dabney protocol (Dabney et al. 2013), as modified by Korlević and colleagues (Korlević et al. 2015), using pre-assembled High Pure Viral Nucleic Acid Large Volume Kit spin columns from Roche instead of a custom MinElute column apparatus.

Library preparation, quality control, and sequencing

Double-stranded DNA libraries were prepared according to a modified Meyer and Kircher (2010) protocol. Individual libraries were prepared from 12.5uL extract each, and intermediate clean-up steps were performed using Qiagen MinElute PCR Purification Kits to retain very short fragments (~30-80 bp). Before amplification, real time qPCR of a small quantity of library (1uL) was performed to assess the number of molecules present and choose the required number of cycles for amplification. All libraries were then double indexed and amplified using Agilent PfuTurbo Cx Hotstart DNA Polymerase. Before sequencing, libraries were quantified using Qubit and TapeStation or Bioanalyzer. The libraries, plus negative controls for each laboratorial step, were then sequenced on an Illumina NovaSeq SP SR100 XP, at the Vienna BioCenter Core Facilities.

Data processing

Raw sequencing data was processed with cutadapt (v2.3) (Martin 2011) to remove library adapters and barcodes from the DNA sequences, allowing for 1 bp overlap and excluding sequences shorter than 18 bp. These sequences were then aligned to the human reference genome hg19, with the mitochondrial genome replaced by the RCRS sequence, using BWA's (v0.7.17-r1188) (Li and Durbin 2009) *aln* command with seeding disabled using -l 1000. We note that the great majority of published ancient DNA data has been aligned to the human genome reference hg19, and therefore we did the same for consistency. Moreover, an alignment to GRCh38 would not be expected to alter our findings, as our results are based on quantitative comparisons of sample metrics, rather than on population genomic analyses. The alignments were then converted to the SAM format using BWA's *samse* command, and subsequently again converted to BAM format with SAMtools *view* (v1.1) (Li et al. 2009) using the quality filter -q 30 and discarding unmapped sequences with -F 4. Duplicated sequences were removed using SAMtools' *rmdup* command. Terminal deamination was assessed using mapDamage (v2.0) (Jónsson et al. 2013), contamination on the haploid X Chromosome of males using hapCon with a threshold of 0.02× or 2000 SNPs (Huang and Ringbauer 2021), and molecular sex was determined by looking at the fraction of sequences aligning to the Y Chromosome compared to the total fraction aligning to both sex chromosomes (Skoglund et al. 2013). To randomly subsample FASTQ files for re-analysis of merged libraries, *seqtk* (github.com/lh3/seqtk) was used. Library complexity curves were estimated using the *lc_extrap* command from *preseq* (v2.0.2) on the merged downsampled BAM files before duplicate removal, with default settings (Daley and Smith 2013). Statistical significance tests were performed in R (v4.2.1) using the function wilcox.test(), and wilcox.exact() when ties were present, and the argument paired=TRUE (R Core Team 2022).

Data access

The raw sequencing data generated in this study have been submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/) under the accession number PRJEB60553.

Competing interest statement

The authors declare no competing interests.

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562
563 **Figure Legends**

564 **Figure 1.** Diagram summarising the 3 main phases of the density separation protocol for ancient
565 DNA: sequential density separation and acquisition of the different interval solutions; washes and
566 repelleting of the bone powders; and DNA extraction following standard methods.

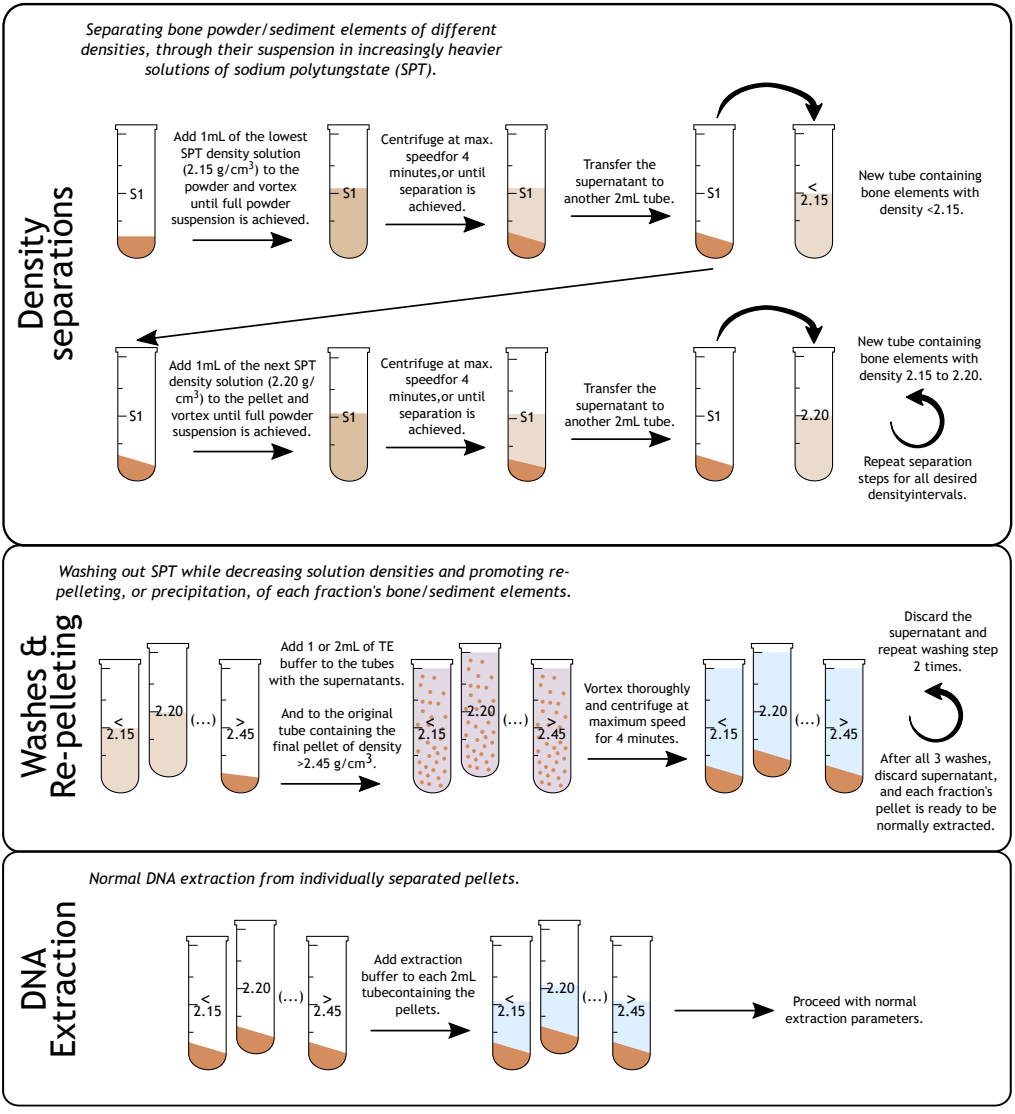
567 **Figure 2.** Distribution (bar plots) and averages (coloured lines) of endogenous DNA percentages after
568 duplicate removal, per interval and per site, using the initial sequencing data ("Sequencing Group 1",
569 **Supplementary Table S1**).

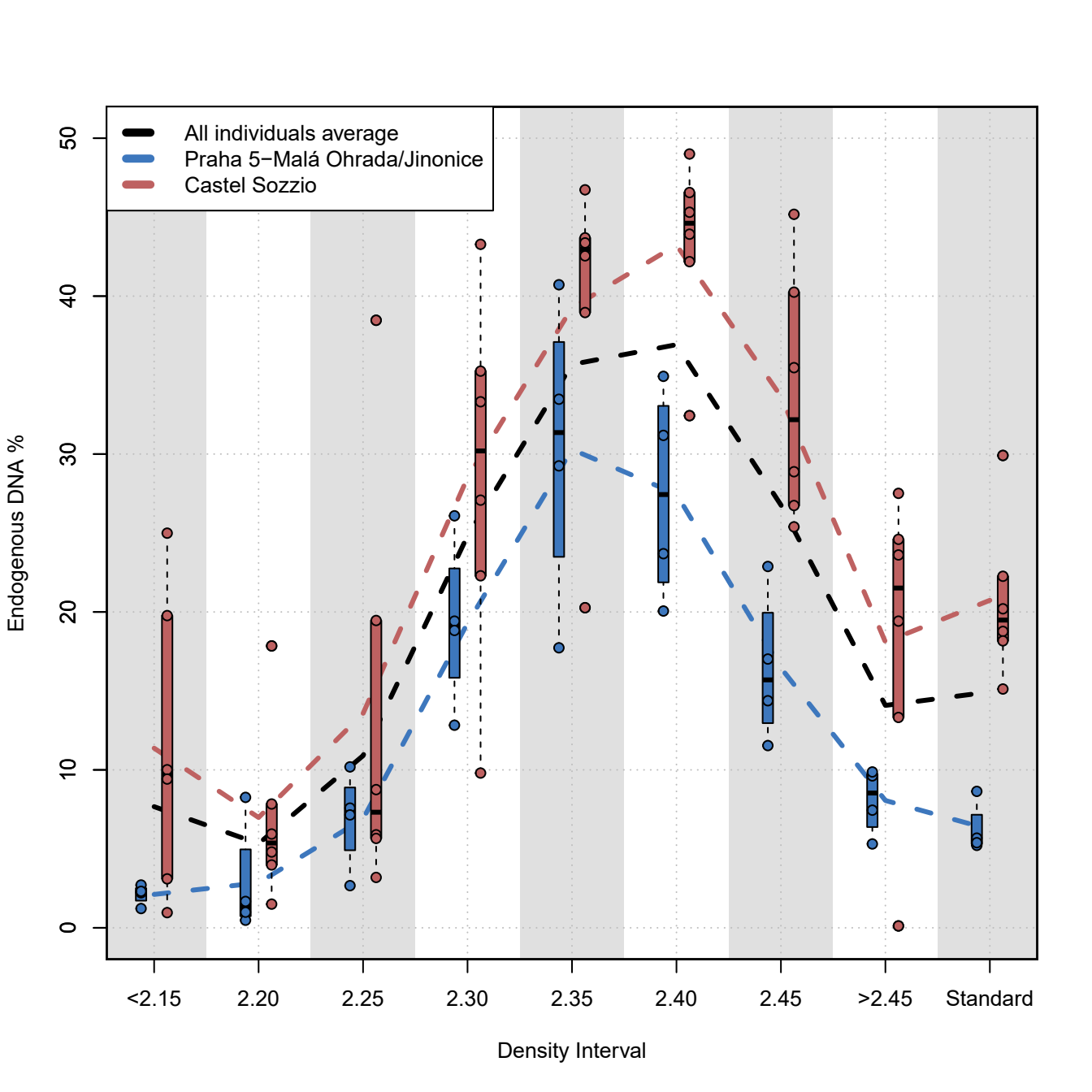
570 **Figure 3.** Comparison of some quality and authenticity metrics between the best SPT interval and
571 the same individual's standard extraction. **A)** Endogenous DNA contents (P-value = 0.001953). **B)**
572 Contamination estimated by hapCon for male individuals (P-value = 0.5469). **C)** Expected distinct
573 reads after deep sequencing as a measure of library complexity, estimated by preseq at a total
574 number of reads of 100 million (P-value = 0.001953). P-values shown in bold denote significance
575 under a threshold of 0.05. Panels A and B use subsampled and normalised total read numbers
576 (**Supplementary Table 2**), whereas panel C uses the full merged data for statistical power
577 (**Supplementary Table 1**).

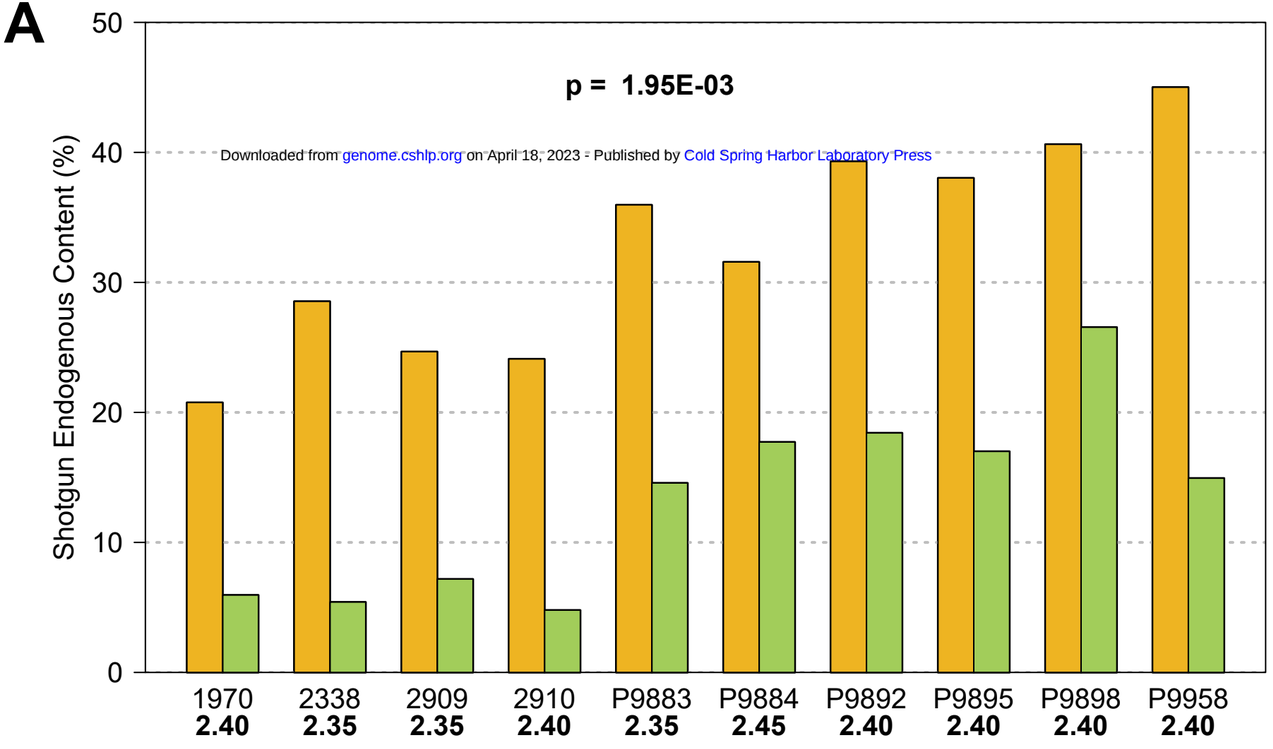
578 **Figure 4.** Fold-increase in endogenous DNA contents as a function of the standard extraction's
579 contents. Sample IDs plotted next to each dot.

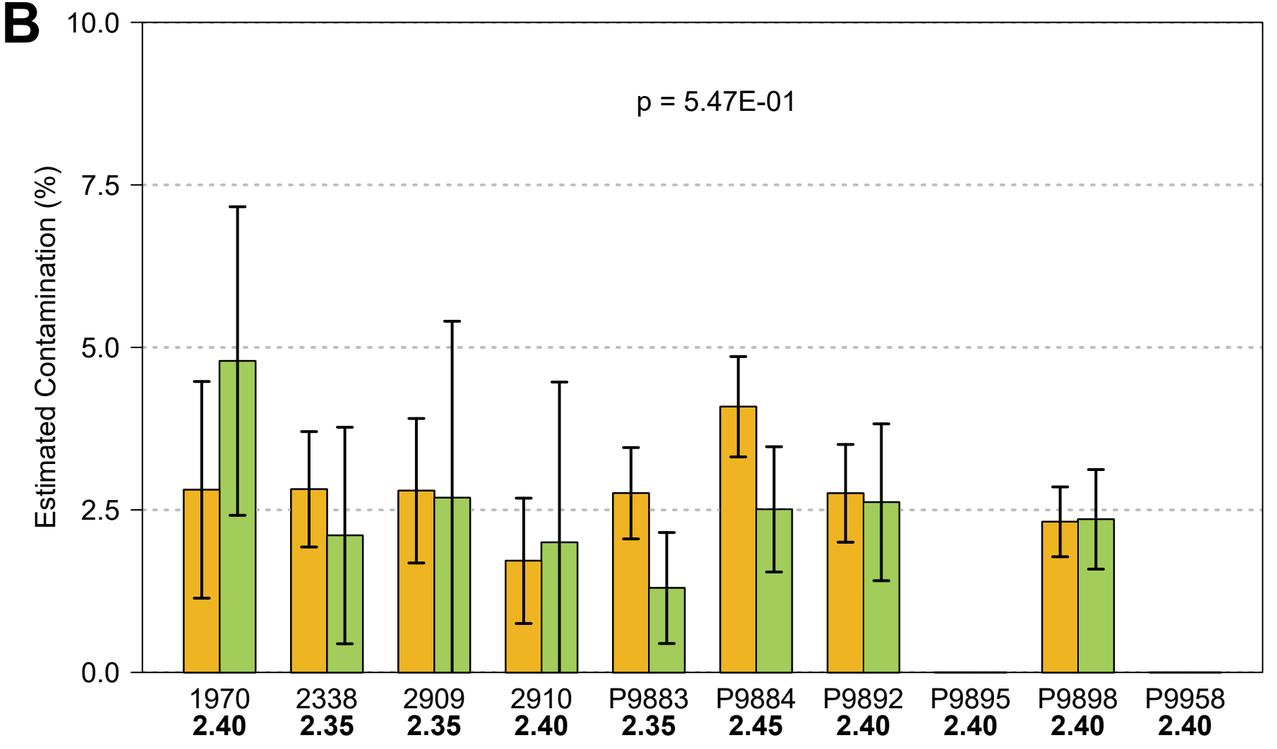
580 **Figure 5.** Yield of expected distinct reads for a theoretically larger sequencing effort, as a measure of
581 library complexity, using the *lc_extrap* function of the software *preseq*. Vertical dotted lines
582 represent the number of reads each sample's best SPT interval and standard extraction were
583 randomly downsampled to. Shaded areas represent 95% confidence intervals. Results shown here
584 for up to 100 million total reads, and in **Supplementary Figure S3** for up to 500 million.

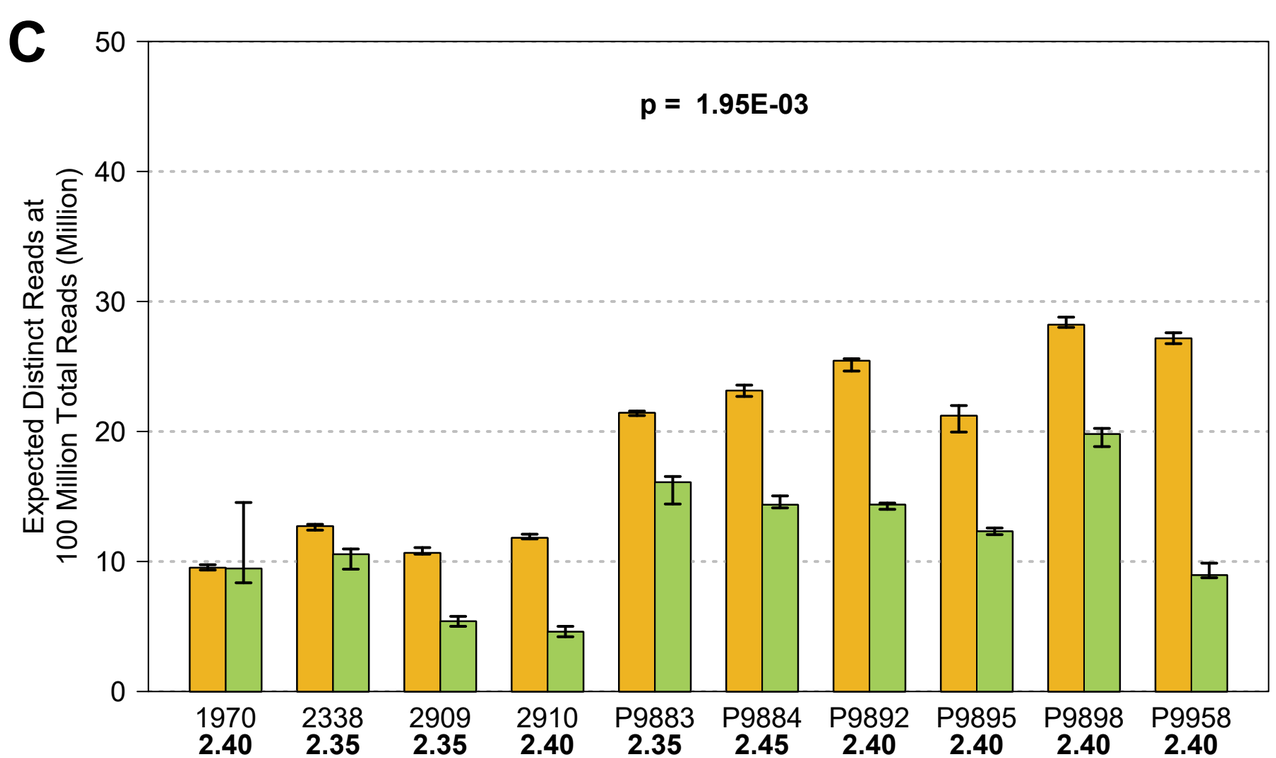
585

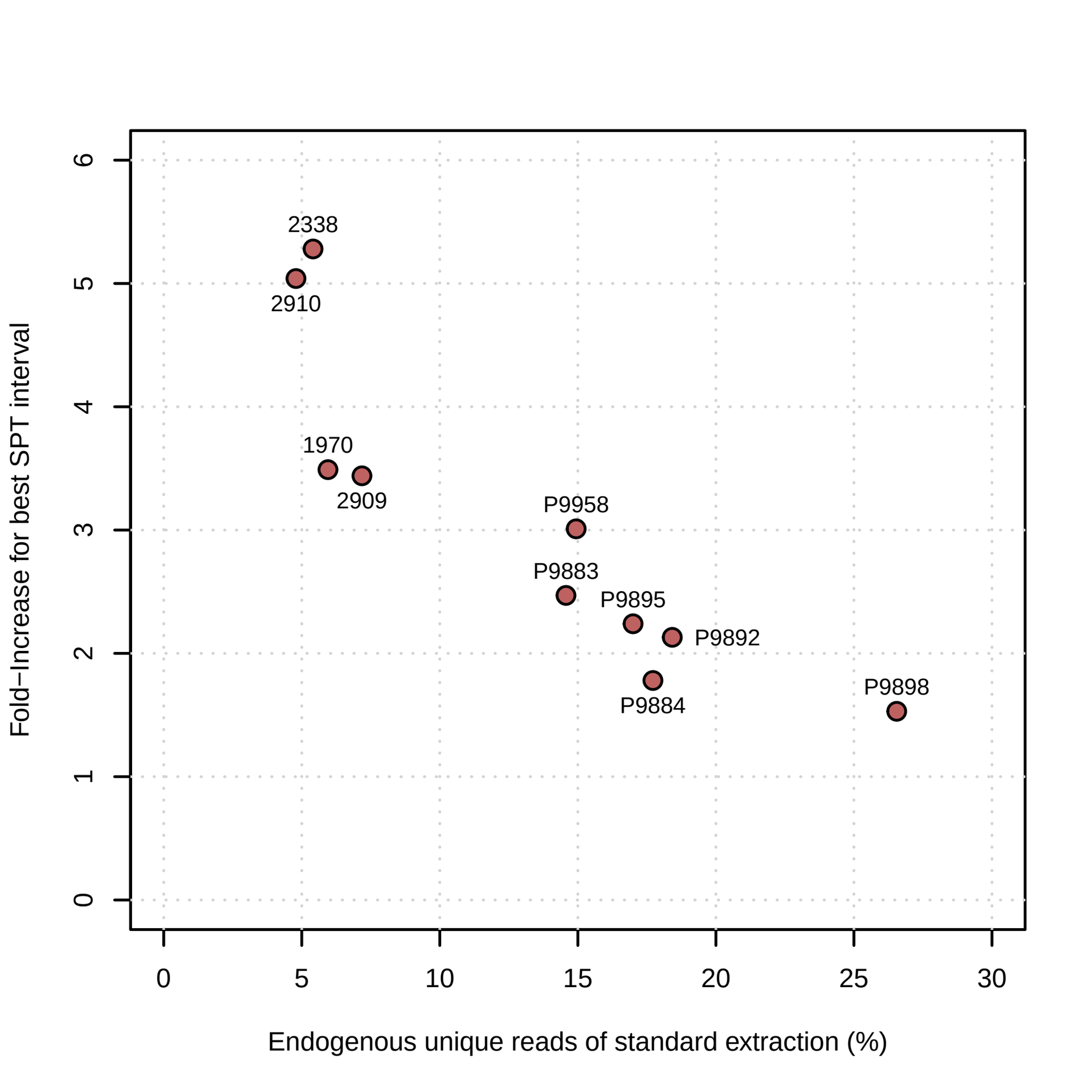

Density separations
Separating bone powder/sediment elements of different densities, through their suspension in increasingly heavier solutions of sodium polytungstate (SPT).
S1
Add 1mL of the lowest SPT density solution (2.15 g/cm3) to the powder and vortex until full powder suspension is achieved.
Centrifuge at max. speedfor 4 minutes,or until separation is achieved.
Transfer the supernatant to another 2mL tube.
< 2.15
New tube containing bone elements with density <2.15.
Add 1mL of the next SPT density solution (2.20 g/cm3) to the pellet and vortex until full powder suspension is achieved.
Centrifuge at max. speedfor 4 minutes,or until separation is achieved.
Transfer the supernatant to another 2mL tube.
2.20
New tube containing bone elements with density 2.15 to 2.20.
Repeat separation steps for all desired densityintervals.
Washes & Re-pelleting
Washing out SPT while decreasing solution densities and promoting re-pelleting, or precipitation, of each fraction's bone/sediment elements.
< 2.15
2.20
(...)
> 2.45
Add 1 or 2mL of TE buffer to the tubes with the supernatants.
And to the original tube containing the final pellet of density >2.45 g/cm3.
Vortex thoroughly and centrifuge at maximum speed for 4 minutes.
Discard the supernatant and repeat washing step 2 times.
After all 3 washes, discard supernatant, and each fraction's pellet is ready to be normally extracted.
DNA Extraction
Normal DNA extraction from individually separated pellets.
Add extraction buffer to each 2mL tubecontaining the pellets.
Proceed with normal extraction parameters.

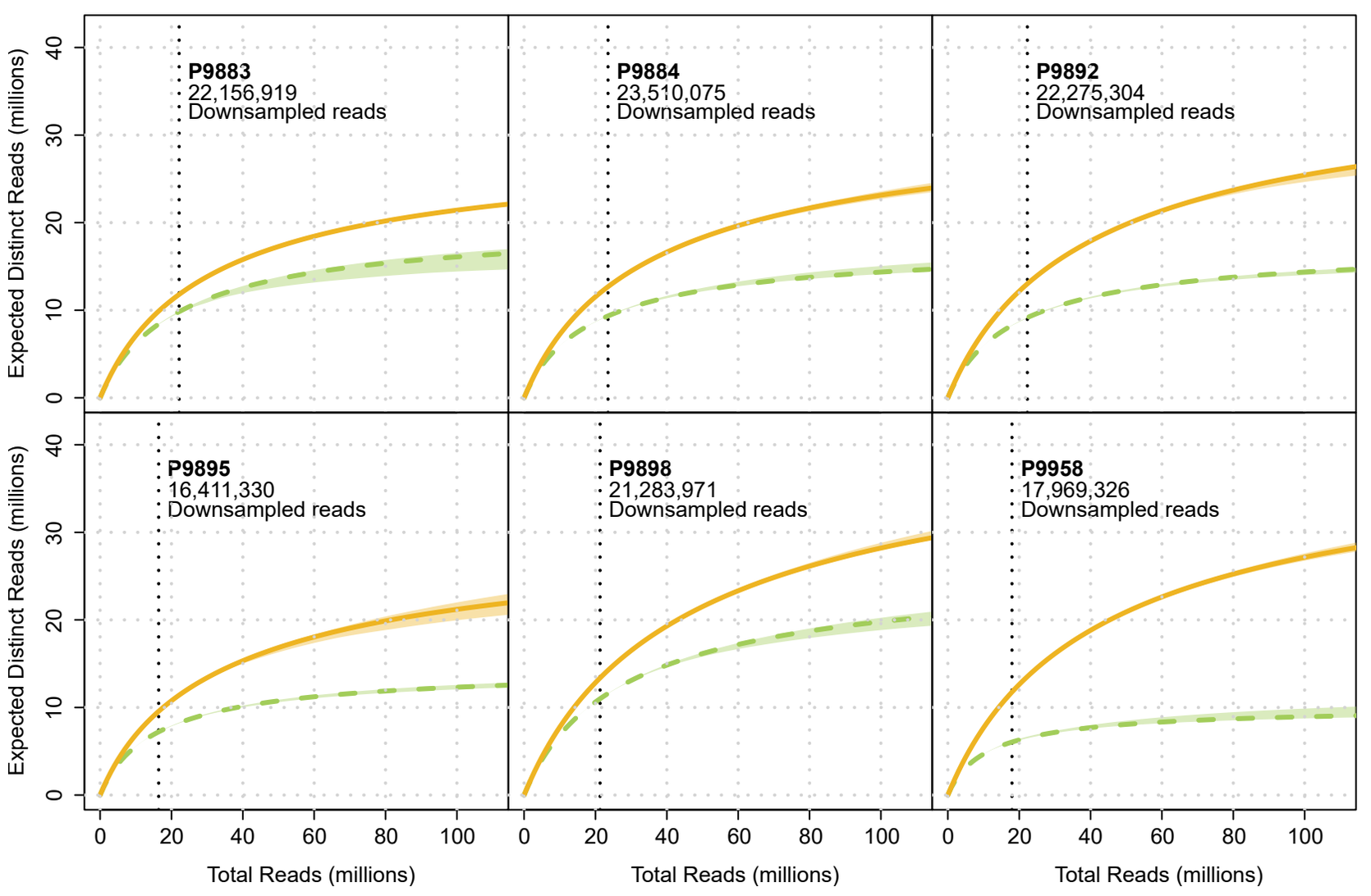

All individuals average
Praha 5−Malá Ohrada/Jinonice
Castel Sozzio
Endogenous DNA %
0
10
20
30
40
50
<2.15
2.20
2.25
2.30
2.35
2.40
2.45
>2.45
Standard
Density Interval

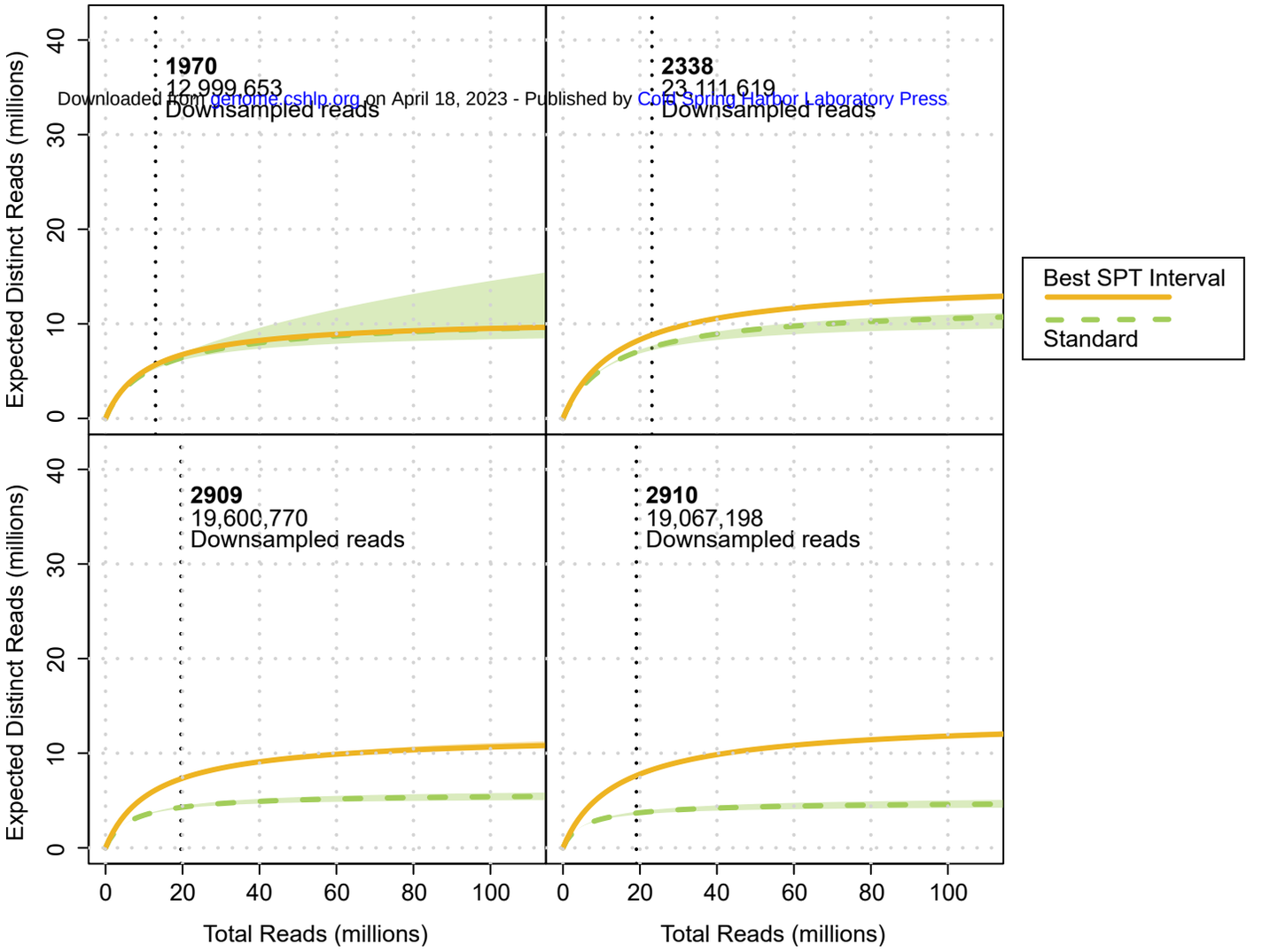







Fold−Increase for best SPT interval
0
1
2
3
4
5
6
2338
2910
1970
2909
P9958
P9883
P9895
P9892
P9884
P9898
0
5
10
15
20
25
30
Endogenous unique reads of standard extraction (%)





Density separation of petrous bone powders for optimized ancient DNA yields

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