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Optimized in-solution enrichment of over a million ancient human SNPs

Roberta Davidson[1*†], Xavier Roca-Rada[1,2*†], Shyamsundar Ravishankar[1], Leonard Taufik[1,3,4], Christian Haarkötter[5], Evelyn Collen[1,6], Matthew P. Williams[7], Peter Webb[1], M. Irfan Mahmud[8], Erlin Novita Idje Djami[8], Gludhug A. Purnomo[1,3,4], Cristina Santos[9], Assumpció Malgosa[9], Linda R. Manzanilla[10], Ana Maria Silva[11,12,13], Sofia Tereso[11,14], Vítor Matos[11,15], Pedro C. Carvalho[2,16], Teresa Fernandes[11,15], Anne-France Maurer[17], João C. Teixeira[1,3,15,18], Raymond Tobler[1,18,19], Lars Fehren-Schmitz[20,21†] and Bastien Llamas[1,3,22,23†]

†Roberta Davidson and Xavier Roca-Rada considered co-lead authors.

†Lars Fehren-Schmitz and Bastien Llamas considered co-senior authors.

*Correspondence: roberta.davidson@adelaide.edu.au; xavier.rocarada@adelaide.edu.au

[1] Australian Centre for Ancient DNA, The Environment Institute, School of Biological Sciences, The University of Adelaide, Adelaide, SA, Australia
Full list of author information is available at the end of the article

Abstract

Background: In-solution hybridization enrichment of genetic markers is a method of choice in paleogenomic studies, where the DNA of interest is generally heavily fragmented and contaminated with environmental DNA, and where the retrieval of genetic data comparable between individuals is challenging. Here, we benchmark the commercial "Twist Ancient DNA" reagent from Twist Biosciences using sequencing libraries from ancient human samples of diverse demographic origin with low to high endogenous DNA content (0.1–44%). For each library, we tested one and two rounds of enrichment and assessed performance compared to deep shotgun sequencing.

Results: We find that the "Twist Ancient DNA" assay provides robust enrichment of approximately 1.2M target SNPs without introducing allelic bias that may interfere with downstream population genetics analyses. Additionally, we show that pooling up to 4 sequencing libraries and performing two rounds of enrichment is both reliable and cost-effective for libraries with less than 27% endogenous DNA content. Above 38% endogenous content, a maximum of one round of enrichment is recommended for cost-effectiveness and to preserve library complexity.

Conclusions: In conclusion, we provide researchers in the field of human paleogenomics with a comprehensive understanding of the strengths and limitations of different sequencing and enrichment strategies, and our results offer practical guidance for optimizing experimental protocols.

Keywords: Ancient DNA, Enrichment, Population genetics, Human paleogenomics

Background

One of the major challenges faced when working with ancient DNA (aDNA) is the high proportion of exogenous DNA contamination present in the DNA extract. This contamination is primarily due to microbes invading the organism post-mortem, present in the

soil where the specimen was buried, or introduced during sample handling and laboratory processes. To counteract this, a method that has become popular is the in-solution enrichment of target genomic regions using pre-designed oligonucleotides as molecular "probes" or "baits." Compared to shotgun sequencing, this technique increases the proportion of target DNA in a sequencing library, lowering sequencing costs required to produce adequate comparable data across individual samples.

In 2012, Patterson and colleagues proposed a molecular bait design for application in human paleogenomic research that made use of a particular ascertainment technique to enable population genetics studies of global human populations over time [1]. The bait design was ultimately restricted to approximately 1.2 million genome-wide SNPs and became known as the "1240k reagent," leading to the generation of thousands of individual genome-wide datasets [2–4]. However, since the original publication of the molecular bait sequences in 2015 [5–7], the legacy 1240k reagent has only been available through a commercial arrangement to a small number of research groups. This presented researchers with the choice of either collaborating with these groups to access the 1240k reagent or using the more expensive deep shotgun sequencing to obtain adequate data compatible with the 1240k SNP loci.

In 2021, two biotechnology firms, Daicel Arbor Biosciences and Twist Bioscience, produced commercial in-solution enrichment kits targeting the same 1240k SNPs plus, in each, an additional set of variants, and made these kits available to every research group [8]. However, recent studies have revealed a strong allelic technical bias in data generated with the Daicel Arbor Biosciences baits [8, 9]. While a comparatively mild allelic bias is also present in the legacy 1240k reagent [8, 9], this has not previously been an issue as all enriched human paleogenomic data were generated with this legacy 1240k reagent and thus co-analyzable.

While many researchers rely on enrichment methods to obtain affordable paleogenomic data, those wary of potential problems arising from the reported biases may prefer the more expensive shotgun sequencing approach. Indeed, because bait binding affinities differ for each allele at a targeted site, all target-hybridization enrichment approaches are expected to have some allelic bias. Accordingly, an important consideration for researchers is whether the realized bias is strong enough to meaningfully affect population genomic analyses and interpretations. Furthermore, paleogenomic research depends upon the comparison of newly generated data to the cumulative set of published genome-wide datasets, making it essential that biases are not introduced when comparing data generated with different methods. Such biases may arise from differences in the assay design or when researchers use untested protocols that deviate from the manufacturer's implementation. For example, some users may wish to pool libraries for input into a single enrichment reaction to decrease the reagent cost per library. However, since the enrichment protocol requires amplifying the libraries using PCR, pooling different libraries might increase instances of index hopping between DNA molecules and introduce biases or cross-contamination between libraries co-enriched within the same reaction [10–14]. Another example involves the choice to perform an extra round of enrichment to increase the target DNA yield, which will likely amplify any biases present in the first enrichment round [9]. While deviations from standard protocols are common, they can introduce variability into potential allelic biases that

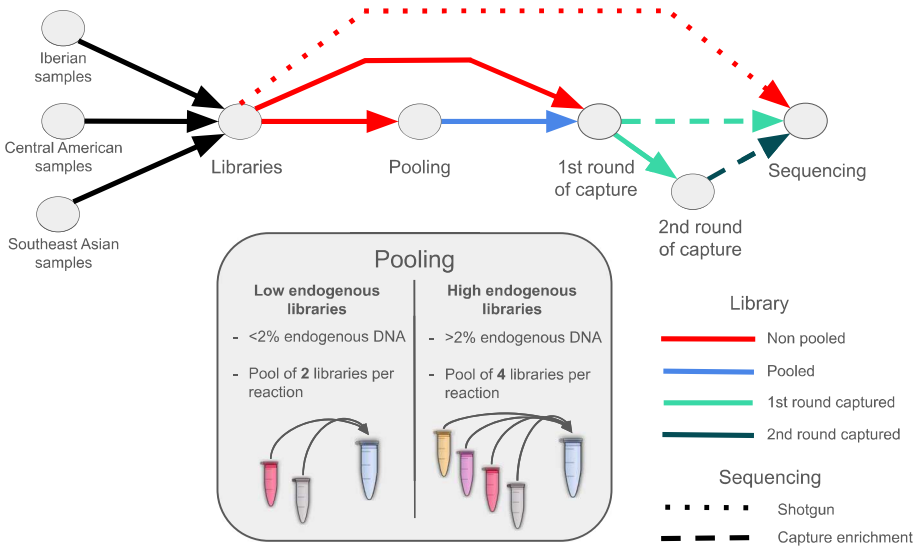

Fig. 1 Twenty-four aDNA libraries, representing samples originating from Iberia, Southeast Asia, and Central America, were selected, all underwent deep shotgun sequencing, and were also input into enrichment experiments. This included six unpooled libraries that were enriched individually, while the remaining libraries were pooled into six distinct reactions: 3 reactions with 2 low endo% DNA libraries each and 3 reactions with 4 high endo% DNA libraries each. All sets of pooled and unpooled libraries underwent one as well as two rounds of enrichment prior to sequencing

may be dangerous for downstream comparative analyses if not thoroughly tested and transparently reported.

Previous work by Rohland and colleagues found no evidence of allelic bias in the Twist assay [8]. However, their study was confined to well preserved West-Eurasian samples. Therefore, in this study (Fig. 1), we aim to benchmark the commercial "Twist Ancient DNA" reagent from Twist Biosciences, using 24 ancient human samples, from four populations from three continents, across a range of endogenous DNA percentages (endo%). We compare deep shotgun sequencing, one and two rounds of enrichment with the Twist Bioscience "Twist Ancient DNA" reagent for cost-effectiveness and allelic biases. We also compare enrichment efficacy and biases between single and pooled library enrichments.

Results

Method comparison

In human paleogenomics, it is common practice to shotgun sequence libraries at a low depth to obtain "screening" data and gather information about library quality (e.g., complexity, endo%) in order to make decisions about further processing. Depending on sample quality and budget, this may lead to deeper shotgun sequencing, target enrichment, or discarding the sample from the experiment. In this study, we selected 24 samples based on the mappable endo% calculated from shotgun screening sequencing data (see Tables 1 and 2. for definitions), with these samples also submitted to deeper shotgun sequencing for the purpose of this study. We subsequently compared read data obtained from the shotgun screening and deep shotgun sequencing steps to better understand how well the shallow shotgun screening

Table 1 Definitions of different read counts

Read count name	Definition
Sequenced	Number of sequenced reads (i.e., paired-end read pairs) in the demultiplexed fastq file before filtering or collapsing
Pre-mapping	Number of reads input to mapping after collapsing, read length, and base quality filtering
Mapped	Number of reads mapping to the target reference genome before further filtering
Filtered	Number of reads mapping to the target reference genome with mapping quality ≥ 25 before further filtering
Dedup	Number of reads retained after filtering mapped reads and removing PCR duplicates

data represents the quality of the DNA sequencing library (Additional file 1: Fig. S1). For all measures of SNP count and endo%, the shotgun screening and deep shotgun results have a strong positive linear correlation with high r^2 values (0.69–0.95). According to the fitted trendlines, deep shotgun sequencing retrieves slightly less SNPs per million reads (slope = 0.86) and a slightly higher endo% than suggested by the screening data (slope = 1.02) (Additional file 1: Fig. S1). These indications are based on the data from only 24 libraries. However, we expect that a larger sample size would likely show the same trends, keeping in mind that samples will likely exhibit inconsistent individual variation between screening and deeper shotgun sequencing.

Comparing the efficacy of deep shotgun sequencing to one and two rounds of Twist enrichment (TW1 and TW2, respectively), we observe that TW2 consistently captured more SNPs per sample in our experiments (Fig. 2A). However, when normalizing the data per million sequenced paired reads, reads into mapping, mapped reads, mapping quality filtered reads, or deduplicated reads (Fig. 2B–F), at least 3 out of 4 libraries with mappable endo% >38% produced less SNPs per million reads after a second round of enrichment. Although two rounds of enrichment consistently yields higher sequenced, mappable, and filtered post-enrichment endo% (Fig. 2G–I), the unique post-enrichment endo% was higher only for libraries with mappable endo% <38% (Fig. 2J). Our results show that two rounds of enrichment may be detrimental to SNP yield for high endo% libraries, possibly because of preferential re-capture of already captured and amplified molecules that ultimately reduces complexity of the library. Nonetheless, overall, *t*-tests revealed significant differences in efficacy metrics between the methods. For the number of target SNPs per million reads sequenced, TW1 resulted in significantly higher recovery compared to SG ($t = 4.03$, $p = 2.07 \times 10^{-4}$), TW2 showed a significant increase compared to TW1 ($t = 2.34$, $p = 0.0238$), and TW2 indicated a highly significant difference compared to SG ($t = 6.52$, $p = 5.00 \times 10^{-8}$). For the mappable endo %, TW1 yielded significantly higher values than SG ($t = 2.30$, $p = 0.0261$), while TW2 had significantly higher values than both TW1 ($t = 2.30$, $p = 0.0261$) and SG ($t = 4.73$, $p = 2.15 \times 10^{-5}$).

Additionally, researchers may wish to identify a lower-bound endo%, below which enrichment would not be expected to work. However, from our results we see a high variability of success in samples with low mappable endo%, with no clear threshold for enrichment success. For example, one sample (mappable endo% = 3.26) increased from 75 to 312 SNPs after two rounds of enrichment, while another sample (mappable endo% = 0.68%) increased from 2068 to 322,197 SNPs (Fig. 2A, Additional file 2: Table S1). Therefore, the decision to apply a lower-bound threshold below which enrichment will

Table 2. Definitions of different quantifications of endogenous DNA percentage in sequencing libraries

Definition	Data	Formula (x100)	Read length filter	Mapping quality filter	Deduplication	Purpose	Comments
Sequenced endo%	Shotgun	$\frac{Mapped}{Sequence}$	$\frac{Y}{N}$	$\frac{N}{N}$	$\frac{N}{N}$	Informs about sample preservation and need for sample enrichment	Estimates are the closest to the true biological endogenous DNA content
Mappable endo%	Shotgun	$\frac{Mapped}{PreMapped}$	$\frac{Y}{Y}$	$\frac{N}{N}$	$\frac{N}{N}$	Informs about sample preservation and need for sample enrichment	Most commonly used in paleogenomic research, but may inflate endogenous DNA estimates when very short reads are abundant
Filtered endo%	Shotgun	$\frac{Filtered}{PreMapped}$	$\frac{Y}{Y}$	$\frac{Y}{N}$	$\frac{N}{N}$	Informs about sample preservation and need for sample enrichment and data robustness	More informative than the above for reads used in downstream analyses
Unique endo%	Shotgun	$\frac{Dedup}{PreMapped}$	$\frac{Y}{N}$	$\frac{Y}{N}$	$\frac{Y}{N}$	Informs about sample enrichment, sample preservation, data robustness, and library complexity	Indicator of library complexity, provides the "useful" proportion of shotgun sequencing data for analyses. Consider in decision to enrich as high duplication, when amplified, can overwhelm unique molecules
Sequenced post-enrichment endo%	Enriched	$\frac{Mapped}{Sequence}$	$\frac{Y}{N}$	$\frac{N}{N}$	$\frac{N}{N}$	Informs about enrichment efficacy	n.a.
Mappable post-enrichment endo%	Enriched	$\frac{Mapped}{PreMapped}$	$\frac{Y}{Y}$	$\frac{N}{N}$	$\frac{N}{N}$	Informs about enrichment efficacy	n.a.
Filtered post-enrichment endo%	Enriched	$\frac{Feltired}{PreMapped}$	$\frac{Y}{Y}$	$\frac{Y}{N}$	$\frac{N}{N}$	Informs about enrichment efficacy and data robustness	n.a.
Unique post-enrichment endo%	Enriched	$\frac{Dedup}{PreMapped}$	$\frac{Y}{Y}$	$\frac{Y}{N}$	$\frac{Y}{N}$	Informs about enrichment efficacy, data robustness, and library complexity retention	Provides the "useful" proportion of enriched data for analyses

not be attempted depends entirely on resources available and the preciousness of the particular samples. Finally, our results highlight the impact of the method used to calculate endo% in the context of enrichments, especially for high endo% libraries.

Cost-effectiveness

One key value of performing library enrichment is the potentially significant reduction in sequencing cost to generate useful data. We tested the cost-effectiveness of deep shotgun sequencing and Twist enrichment using one or two enrichment rounds by applying all three approaches on the same set of 24 samples. While the specific costs are unique to our experiment, it is clear that the Twist enrichment (1 or 2 rounds) is more cost-effective per SNP than shotgun sequencing. This advantage is greatest for libraries with low mappable endo% (Fig. 3). Further, our fitted logarithmic model predicts that 2 rounds of enrichment was more cost-effective per SNP than 1 round only for libraries with mappable endo% < 20% (Fig. 3). Finally, we computed a generalized linear regression to compare the cost-effectiveness of TW1 and TW2 against shotgun sequencing, controlling for the starting endo% of the library. Results indicate TW2 was significantly more cost-effective than shotgun ($p = 0.041$, Additional file 2: Table S4) and TW1, although non-significant, was similar ($p = 0.055$, Additional file 2: Table S4).

We note, however, that this threshold depends on the relative costs of enrichment and sequencing and thus should be considered as a guide rather than a definitive value for future studies. We also compared the relationship between cost per SNP and the number

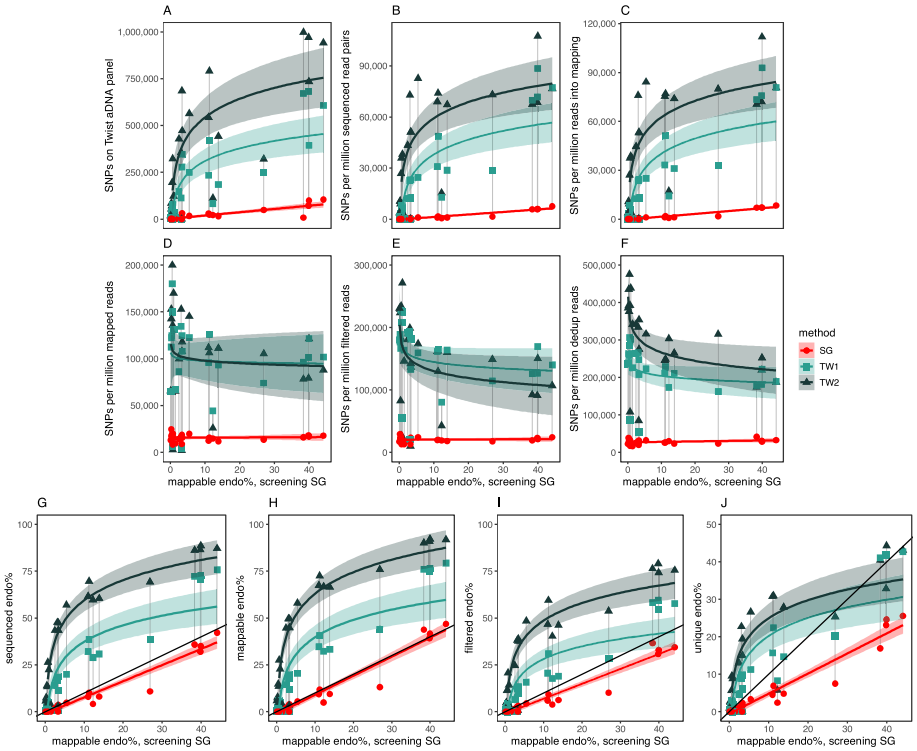

Fig. 2 Enrichment efficacy of deep shotgun sequencing (SG) or Twist enrichment (either one or two rounds, i.e., TW1 or TW2, respectively) in relation to the mappable endo% from the screening shotgun data (x-axis). Enrichment efficacy was measured using **A** total on-target SNPs per sample, **B** SNPs per million sequenced read pairs, **C** SNPs per million reads going into mapping, **D** SNPs per million mapped reads, **E** SNPs per million filtered reads, passing filter for MAQ > 25, **F** SNPs per million unique reads, **G** sequenced post-enrichment endo%, **H** mappable post-enrichment endo%, **I** filtered post-enrichment endo%, and **J** unique post-enrichment endo%. Point shape and color corresponds to the method. Gray vertical lines connect points showing the three methods performed on the same library. The solid lines and shaded areas show fitted linear regression models (shotgun) or logarithmic transformed linear regression models (Twist enrichments) as appropriate. The black line in **G**–**J** shows y = x

of retrieved target SNPs (Fig. 4), which revealed that Twist enrichment obtained more total and relative target SNPs than shotgun sequencing. Additionally, for the majority of samples, 2 rounds of enrichment generated more SNPs and was more cost-effective than a single round (Fig. 4). We note that extra time is required to perform enrichments and acknowledge that labor costs were not included in this analysis as this is a highly variable factor in academia, and realistically, the choice to conduct enrichment also includes consideration of project and personnel contract deadlines, etc. Additionally, we note that the cost of enrichment and sequencing might vary significantly depending on pricing agreements with manufacturers, distributors, and service providers, and changing costs over time. We have therefore made Fig. 3 available as an interactive app where users can input their own costs of sequencing and the Twist enrichment kit to and encourage other research groups to use the tool to carefully consider their own circumstances when determining the number of rounds of enrichment for their study.

Allelic bias

To measure allelic bias, we performed f_4 statistics of the form f_4 (Mbuti, Pop1.MethodA; Pop2.MethodA, Pop2.MethodB), where the outgroup were Mbuti individuals from the Allen Ancient DNA Resource (AADR) [15, 16] and Pop1 and Pop2 were one of East Iberia, West Iberia, Central America, or Southeast Asia and the methods were one of shotgun, TW1, or TW2. Here, given that the same population is present in the right hand pairing of the equation and an outgroup to Eurasian and American populations placed in the first position, significant deviations from $f_4 = 0$ are indicative of allelic bias between the tested Pop1.MethodA left population and one of the Pop2 right populations. The bias may signal differential allelic affinities across the assay methods (Fig. 5) [9].

In all tests, the absolute Z score is less than 3, and the majority are less than 2, indicating that none of the observed f_4 statistics is significantly different from zero. Accordingly, we find no evidence for significant allelic assay bias from the Twist enrichment, whether between one and two rounds of enrichment, or between any enrichment and shotgun data, providing support for the combined analysis of Twist and whole genome sequencing data (Fig. 5, Additional file 2: Table S5).

Impact of pooling

A concern when pooling libraries for enrichment is that adding more libraries into the reaction may decrease the yield from each individual library. To evaluate this, we compared the number of SNPs obtained per million reads against the mappable endo% to see if pools with fewer libraries generated more SNPs per library (Additional file 1: Fig. S2). Point distributions for the three types of pool overlap and linear trendlines for pooled libraries suggest higher SNP yield than in unpooled libraries.

Another unknown regarding the pooling of libraries for enrichment is how equitably the enrichment reaction captures targeted SNPs from each of the libraries, and if any inequities would be amplified by two rounds of enrichment. To investigate this, we compared various read counts from successive stages of data processing, between each library in every pool (Fig. 6). The proportion of the total reads contributed by each library in a pool remains quite consistent between TW1 and TW2. In most pools, the read proportions are similar across the different read counts, with the

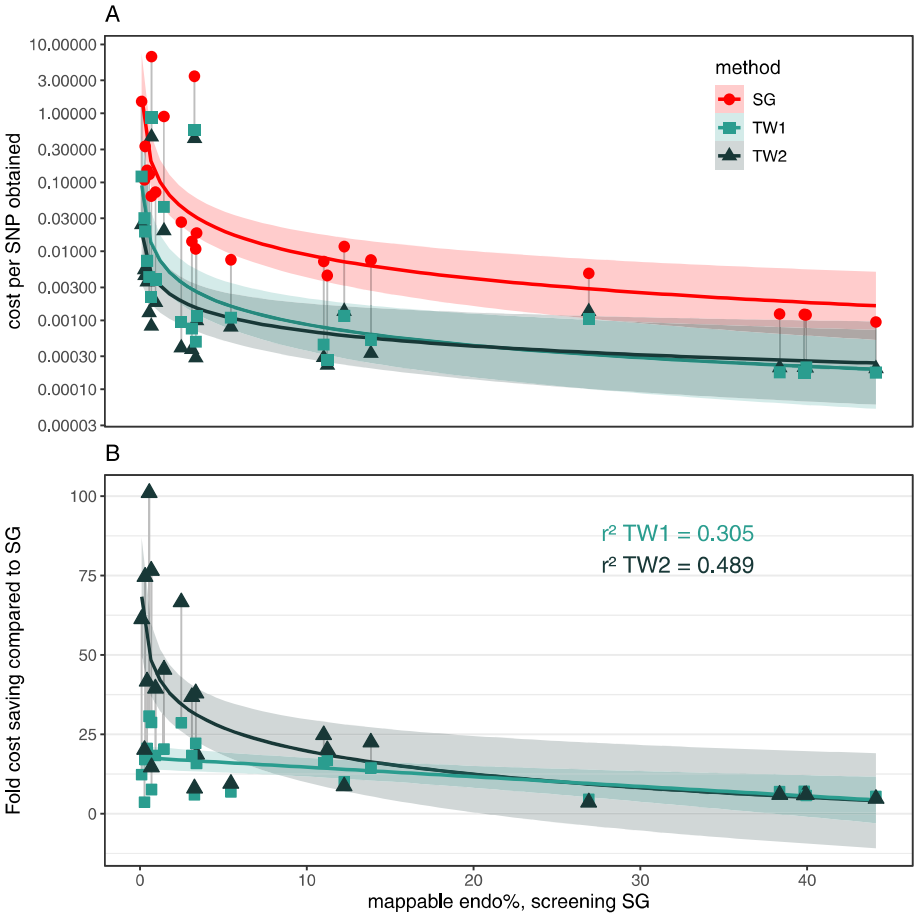

Fig. 3 **A** Cost per SNP (AUD) obtained from shotgun sequencing and Twist enrichment methods as a function of the mappable endo%. The y-axis is log10 transformed. **B** Relative fold cost saving per SNP (AUD) obtained from TW1 and TW2 compared to deep shotgun sequencing. The r^2 values represent the correlation value of the data points with the fitted model trendline. Point shape and color corresponds to the method. Gray vertical lines connect points showing the methods performed on the same library. Linear or log10 transformed linear models are fit to each method (solid lines), with 95% confidence intervals (shaded areas). This figure is also available as an interactive app where users can input different costs of the Twist enrichment kit or sequencing to predict how the savings will change with different prices. See https://roberta-davidson.github.io/Davidson_etal_2024-Twist/ for the interactive plot

biggest observable change being between the reads into mapping and mapped reads when mappable endo% is low (Fig. 6). This is likely because the pooling calculations are based on DNA concentration of the library rather than the endo% of each library. Of note, the high mappable endo% library in the HE4 pool that drops out after mapping (Fig. 6, bottom sub-bar in pink in HE4 facets) is characterized by a high rate of duplication.

Another concern when pooling libraries is "cross-contamination" of samples due to dual index hopping between DNA molecules from different libraries. Therefore, we first calculated the pairwise rate of single index hopping by counting all possible combinations of the library indexes included in our experiment. These pairwise hopping rates were evaluated separately for unpooled libraries and each pool size (2 and 4 libraries), knowing that index hopping observed in unpooled libraries can only occur

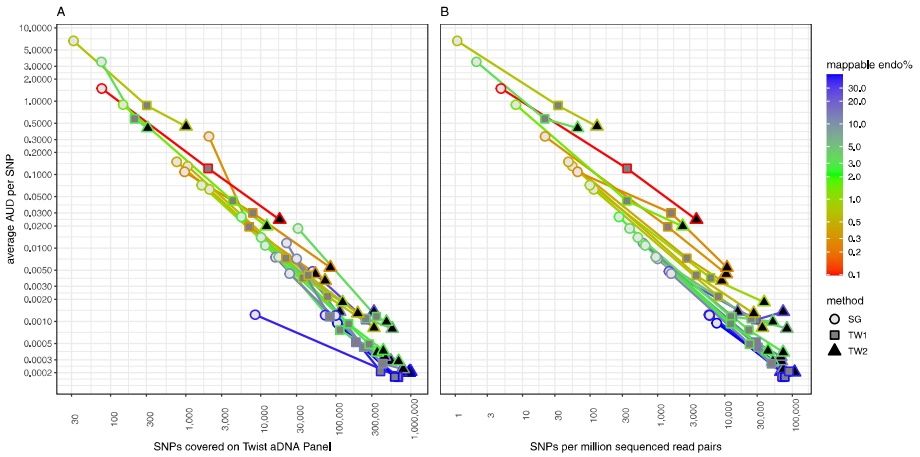

Fig. 4 Cost per SNP in relation to SNP coverage (x-axis) and mappable endo% measured from screening shotgun data (colors) for deep shotgun sequencing and enrichment methods (shapes). **A** Total SNP coverage on Twist ancient DNA panel and **B** SNPs per million sequenced read pairs. Lines connect the same library across the three methods. Plot axes and color scale are log10 transformed

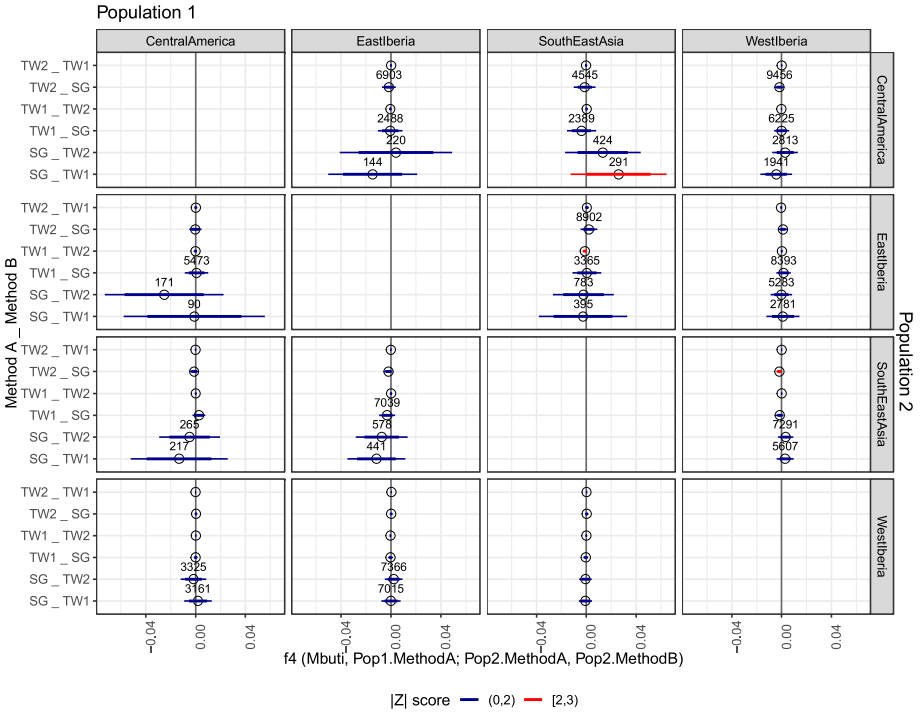

Fig. 5 f_4 statistics of the form f_4 (Mbuti, Pop1.MethodA; Pop2.MethodA, Pop2.MethodB). Pop 1 and 2 refer to the geographic population used in each statistical calculation and are faceted horizontally and vertically, respectively. Method A and B refer to the comparison of two of the three methods (SG = shotgun, TW1 = 1 round of Twist enrichment, TW2 = 2 rounds of Twist enrichment); the methods analyzed are annotated on the left y-axis. Thick and thin error bars represent 2 and 3 standard error deviations (s.e.), respectively. Tests where $|Z| > 2$ are colored red, no test returned $|Z| > 3$. Tests with < 10,000 SNPs are annotated with the number of SNPs

during sequencing. We performed *t*-tests and found significant difference in single index hopping rate for pooled and unpooled libraries (p values: $p = 2.55\text{e}-03$ for 2 vs 4 library pools, $1.93\text{e}-03$ for 2-library pools vs unpooled libraries, and $1.00\text{e}-09$ for

4-library pool vs unpooled libraries) (Fig. 7). In contrast, *t*-tests showed no significant difference in single index hopping rate between one and two rounds of enrichment ($p = 0.93$ and $p = 0.76$ for 2 and 4 library pools, respectively) (Additional file 1: Fig. S3). Taking the observed mean pairwise single index hopping rate of ~0.013 for pairs in 2-library pools (Fig. 7), we estimate the expected proportion of dual index hopped reads is ~0.00016, or 160 reads per million, assuming that indexes hop independently. Then, following the same assumptions, the observed mean pairwise single index hopping rate between a pair of libraries in a 4-library pool is ~0.0059 (Fig. 7), and given that there are six pairs of libraries in a 4-library pool, the estimated proportion of dual index hopped reads between any two libraries in a 4-library pool is $\sim 0.0059^2 \times 6 = \sim 0.00021$, or 208 reads per million.

Furthermore, to investigate the prevalence of dual index hopping in our experiment, we applied several tests for sample cross-contamination, given that a dual index hopping event would appear in the sequencing data as contamination from another sample. We used HaploCheck to measure mitochondrial contamination, ANGSD and HapConX to measure contamination of the X chromosome in males (Additional file 2: Table S2), and DICE to estimate autosomal contamination, with all results reported in Additional file 2: Table S3.

Results from HaploCheck revealed the absence of detectable contamination in any sample (Additional file 2: Table S3). However, five samples were reported with

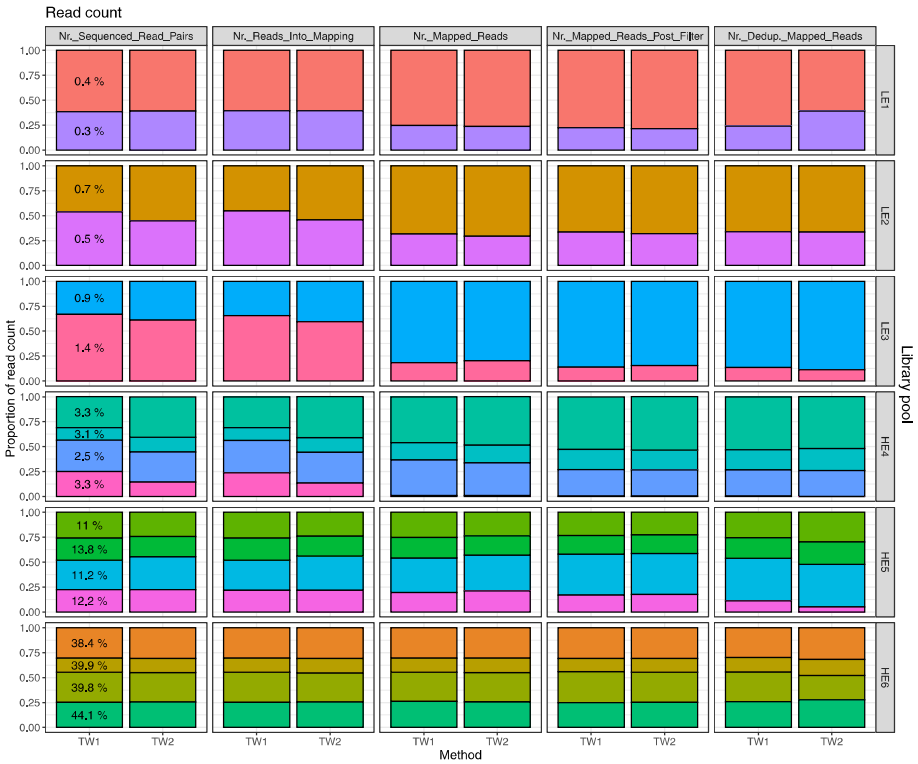

Fig. 6 Equity of library enrichment in pooled reactions. Library pools are ordered from low to high endo% from top to bottom. Color denotes one library and each library is labeled in the first column with the mappable endo% calculated from shotgun screening sequencing. Panels show from left to right: number of sequenced read pairs, number of reads input to mapping, number of reads mapped, number of mapped reads passing mapping quality (≥ q25), and deduplicated mapped reads

artifactual "heteroplasmies" by HaploCheck, raising the possibility of index hopping contamination. Among the affected libraries, three (TW024_NA_TW2, TW015_HE6_TW2, and TW016_HE6_TW2) had fewer than 1000 reads mapping to the mitochondrial genome, presenting a challenge for haplogroup assignment. Notably, despite all three libraries being subjected to both single and dual rounds of enrichment, heteroplasmy was not detected in TW1. Of the remaining two affected samples, one library (TW006_HE6) was impacted in both TW1 and TW2, while the other (TW008_LE1) was once again only impacted in TW2. None of these artificial heteroplasmy loci was considered in haplogroup determination and when read pileups were inspected visually, they did not present a challenge for consensus sequence calling. Interestingly, we noticed unexpectedly low coverage for mitogenomes in Twist-enriched data, suggesting that increasing the number of mitochondrial probes during enrichment would be beneficial. Additionally, there was a decrease in mitochondrial genome coverage in TW2, indicating fewer unique reads and decreased complexity. Consequently, minimizing PCR cycles is recommended in both the pre-enrichment overamplification and enrichment PCRs.

X-chromosome contamination was estimated in the 18 male libraries in our experiment with ANGSD and HapConX. In the ANGSD results, all pools exhibit zero contamination for male samples whereas female samples are reported as contaminated, which is expected given the presence of two different X chromosomes and serves as a positive control. The reported values are consistent with the expected levels of heterozygosity observed in females. We caution that some contamination values reported by ANGSD

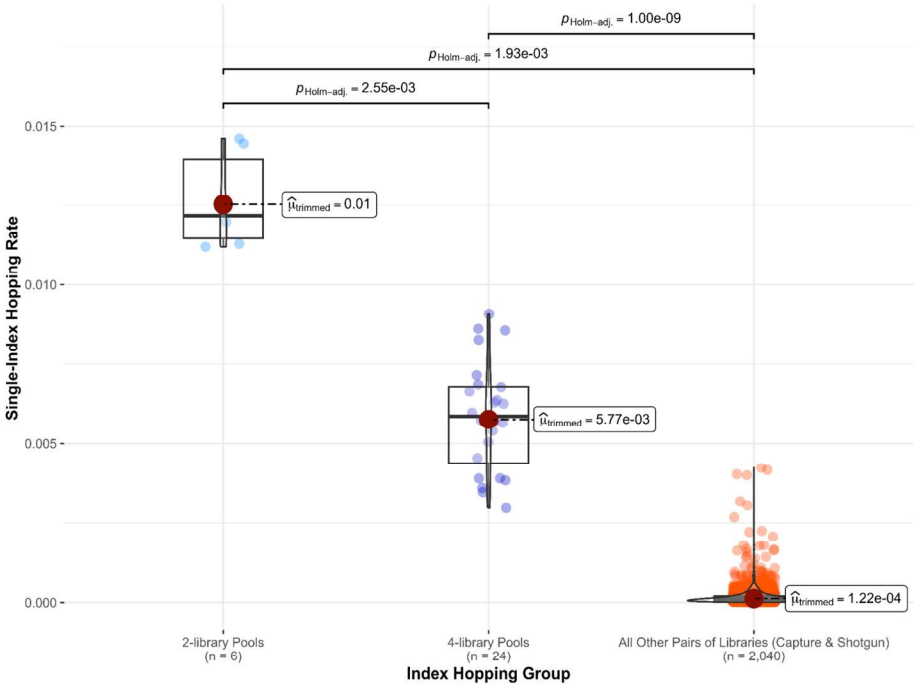

Fig. 7 Pairwise rate of single index hopping between every pair of libraries, categorized into (left to right) 2-library pools, 4-library pools, and all other pairs of libraries. Each category is annotated with the number of pairs (*n*). The hopping rate was calculated as the proportion of reads with a hopped index combination over the sum of reads from every possible index combination within the pair

are for samples with a total absence of X chromosome SNPs and are therefore ignored. HapConX reported extremely low estimated contamination for all samples, the highest being ~0.037. Lastly, we ran DICE to estimate autosomal contamination, utilizing the different demographic origin of the samples; however, this was only achievable for one pool (HE6; having one Central American individual and three Iberian individuals) due to poor data quality of low-endo% pools and a lack of relevant publicly available reference populations for our Southeast Asian samples. The DICE output converged upon a low contamination estimate of ~4.55e −05 and 4.99e −04 for TW1 and TW2, respectively (Additional file 1: Fig. S4).

Discussion

The findings of this study offer valuable insights into optimizing enrichment strategies in human paleogenomics through the use of shotgun screening and pooling strategies for targeted enrichment. The positive linear relationship observed between shallow and deep shotgun sequencing data for SNP count and endo% underscores the reliability of shallow shotgun screening as a preliminary assessment tool for downstream analyses and decisions concerning aDNA library enrichment.

The high cost of deep shotgun sequencing compared to enrichment methods in contemporary paleogenomics is an important factor in favoring the latter approach, whether through the application of one or two rounds of enrichment. Our analyses show that, in general, it is most cost-effective to perform two rounds of enrichment. Although a decrease in SNPs per million sequenced reads is observed in high endo% libraries that were subjected to a second round of enrichment, suggesting that the benefits of two rounds of enrichment are dependent on sample quality. In general, it is expected that enrichment will increase the proportion of DNA in the library that is targeted by the bait set, and that two rounds of enrichment will increase this proportion even more, although this increased endogenous DNA includes an increase in duplicates, given that every enrichment includes a PCR amplification. One possible explanation for our observed decline in efficiency at high endo% is the preferential re-capture of already enriched molecules during the second round of enrichment. Since hybridization-based capture methods rely on probe binding, molecules that have already been enriched in the first round are more likely to be captured again, leading to a higher proportion of duplicate reads. This effect is more pronounced in libraries with high endogenous DNA content, where the target molecules are already well-represented in the sequencing pool. Additionally, PCR amplification prior to sequencing further exacerbates this issue, as it increases the redundancy of over-represented fragments, thereby reducing the fraction of unique reads. Comparing mappable and unique enriched endo% calculations shows that two rounds of enrichment for high mappable endo% libraries may result in sequencing a majority of duplicate reads, reducing the proportion of unique reads sequenced as well as the corresponding target SNP yield and mitochondrial genome coverage. This highlights the need for reducing the number of PCR cycles and/or opting for a single round of enrichment for libraries with high endo%—our study suggests the latter applies to libraries with mappable endo% > 38%, possibly > 27% (our experimental design did not include libraries with mappable endo% in the range 27–38%).

Our assessment of allelic bias using f_4 statistics yielded reassuring results, suggesting the absence of observable assay bias introduced by the Twist Bioscience "Twist Ancient DNA" reagent. This finding further consolidates the reliability of this reagent in producing unbiased results for paleogenomic analyses, providing a notable improvement compared to previously reported paleogenomic enrichment data generated with the legacy 1240k reagent and the Daicel Arbor Biosciences Expert Human Affinities Prime Plus enrichment kit [8, 9].

In addition to targeted probe-based enrichment strategies, whole-genome capture (WGC) is a relevant alternative. Previous studies, such as [17], have demonstrated that WGC is an efficient method for obtaining unbiased genomic data from aDNA samples. Unlike targeted enrichment, which relies on the hybridization of probes specific to genomic regions of interest, WGC allows the capture of sequences across the entire genome, minimizing allelic bias and increasing the representation of rare variants.

Moreover, our exploration of the effects of pooling several libraries into a single enrichment reaction also yielded reassuring outcomes. We suggest that pooling up to four libraries does not have a substantial impact on SNP yield compared to single library reactions. We investigated the potential for cross-contamination due to dual index hopping between molecules originating from different libraries. Even though there is a significant difference in single index hopping rates between pooled and unpooled libraries, the undetectability of dual index hopping through the calculation of contamination estimates, coupled with its extremely low estimated occurrence rates, underscores the reliability and cost-effectiveness of the pooling approach.

Conclusions

In summary, our study emphasizes the efficacy and cost-efficiency of using shallow shotgun screening followed by pooling libraries into target enrichment reactions for human paleogenomics. We provide a cost–benefit analysis for a wide range of endogenous DNA preservation levels, which researchers can use to design their studies more effectively.

Methods

Facilities

Pre-amplification experiments were performed at the Australian Centre for Ancient DNA (ACAD)'s ultra-clean laboratory facilities following rigorous laboratory procedures to minimize contamination and ensure high standards of quality for the genetic data [18, 19]. All post-amplification experiments were completed in standard molecular biology laboratories at the University of Adelaide and subsequent bioinformatics workflows executed on the University of Adelaide's High Performance Computing service.

DNA extraction and library preparation

Skeletal remains from 24 ancient humans sourced from Iberia, Central America, and Southeast Asia were used for these experiments. Prior to DNA extraction, skeletal samples were sterilized using ultraviolet light (UV), bleach, and ethanol to minimize surface contamination. Approximately 0.1 g of bone powder was used for DNA extraction. Ancient DNA molecules were retrieved using a method optimized for degraded DNA [20] and partially UDG-treated [21] double-indexed double-stranded DNA libraries

were subsequently generated [22]. Quality control and quantification steps were completed using Qubit (Thermo Fisher) and TapeStation (Agilent) prior to overamplification and enrichment or shotgun sequencing.

Library enrichment

Libraries were over-amplified in order to reach the 1000 ng needed for enrichment per pool. For each library, the PCR reaction mix consisted of 5–10 µl of library, 25 µl of KAPA HiFi HotStart ReadyMix (Roche), 5 µl each of 10 µM IS5 and IS6 primers [21], and ultrapure water in a total volume of 50 µl. PCR amplification was performed with an initial denaturation and polymerase activation at 98 °C for 2 min, 15 cycles of 98 °C for 20 s, 56 °C for 30 s, 72 °C for 45 s, and final extension at 72 °C for 5 min. DNA purification was performed using 1.2 × AmpureXP beads with two 80% ethanol washes, and the DNA was eluted in 30 µl of water.

To compare the performance and cost-effectiveness of single enriched libraries versus pooled enriched libraries, we prepared (i) six reactions, each consisting of 1000 ng of DNA from a single library, each exhibiting varying endogenous DNA percentages as determined from prior screening shotgun sequencing runs; (ii) three reactions consisting of two low endogenous DNA pooled libraries; and (iii) three reactions consisting of four high endogenous DNA pooled libraries (Figs. 1 and 8, Additional file 2: Table S1). While each enrichment reaction contained a final quantity of 1000 ng of DNA, the amount of DNA required per library was reduced due to pooling, allowing for a decrease in PCR cycles to avoid overamplification and maintain library complexity. Library pooling was calculated from total mass DNA quantification of each library, rather than endogenous content calculated from the shotgun screening data. Moreover, pools of two libraries were configured to include Iberian and Central American or Southeast Asian samples, and pools of four were configured to include three samples of Iberian origin and the remaining sample having either Central American or Southeast Asian demographic origin. This enabled the evaluation of cross-contamination created by dual index hopping across DNA fragments from libraries with different demographic origins in pooled library enrichments.

Enrichment was performed using the Twist Bioscience "Twist Ancient DNA" reagent following the manufacturer's protocol (Fig. 8). For each input pool, we independently performed one and two rounds of enrichment (labeled as TW1 and TW2, respectively) in order to compare performance and cost-effectiveness. The post-enrichment PCR amplification was performed using KAPA HiFi HotStart ReadyMix (Roche) and IS5 and IS6 primers as described above, with a 98 °C initialization for 24 s, 15 cycles (1 st round) or 7 cycles (2nd round) of 98 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, and a 72 °C final extension for 60 s. DNA purification and library quality control and quantification were performed as described above.

After the first round of enrichment, we observed heteroduplex formation in some library profiles. Heteroduplexes arise when library fragments with slight mismatches anneal to each other during the cooling phases of PCR, leading to the formation of hybrid molecules. These are detectable as peaks of longer molecules when observed on TapeStation (Agilent). To resolve this for libraries proceeding directly to

sequencing without a second round of capture, we performed a reconditioning PCR, which is just a single cycle of PCR. Libraries were concentrated down to 5 µl and mixed with 10 µl of Herculase Buffer (Agilent), 5 µl of 2.5 nM dNTPs, 1 U of Herculase II Fusion (Agilent), 1 µl each of 10 µM IS5 and IS6 primers, and ultrapure water in a final volume of 50 µl, then reconditioned with one cycle of 95 °C for 2 min, 58 °C for 2 min, and 72 °C for 5 min. This step denaturates the library by separating heteroduplexes and amplifying correctly and minimizes issues with downstream quantification and sequencing. In contrast, libraries undergoing a second round of capture did not require reconditioning PCR. The DNA denaturation step at the beginning of the second capture round effectively resolved any heteroduplexes, and the lower number of cycles in the second round's post-enrichment PCR (e.g., 7–10 cycles) prevented their reformation. DNA purification and library quality control and quantification were performed as described above.

Sequencing

All shotgun and enriched libraries were sent for sequencing using a NovaSeq 6000 System with a 2 × 100 bp SP Flow Cell in XP mode at the Kinghorn Centre for Clinical Genomics (Sydney, NSW, Australia).

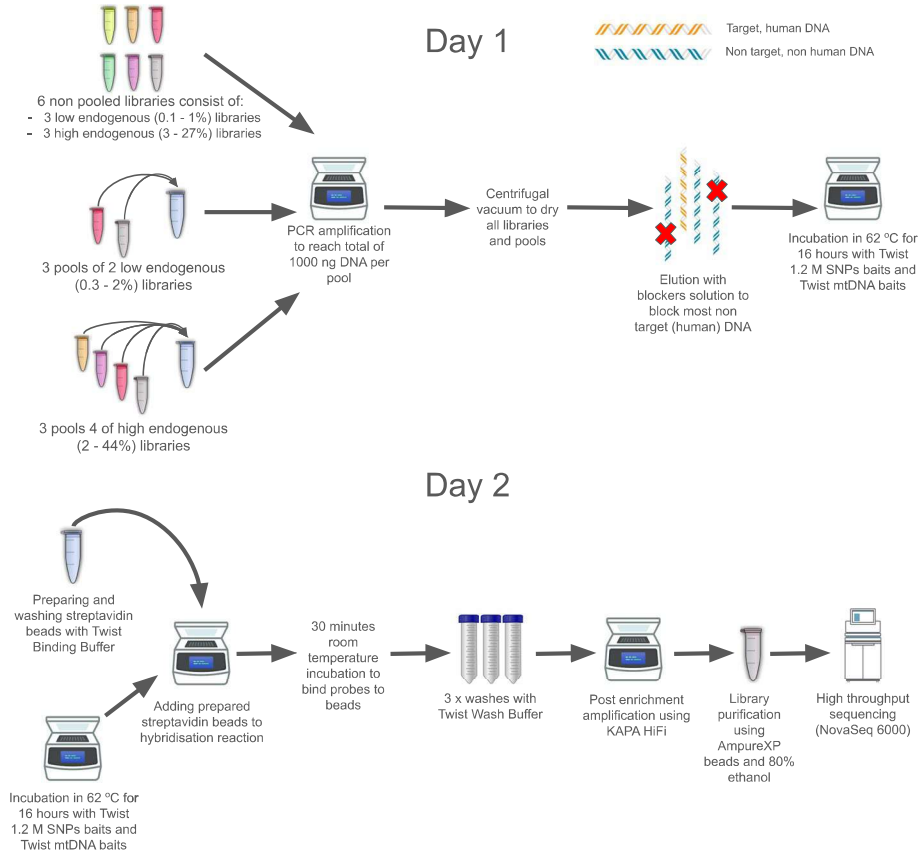

Fig. 8 Detailed workflow of the experimental design for Twist enrichment laboratory protocol used in this study, showing the steps conducted on day 1 and day 2, with the hybridization incubation overnight between the days

Data processing

Raw data were processed with the aDNA analysis workflow package nf-core/eager version 2.4.6 [23]. Merged read mates were mapped to the GRCh37 d5 reference genome using *bwa aln* with parameters *-l 1024 -n 0.01 -o 2* (20). Two nt were trimmed from the terminal ends of all retained reads using the *trimBam* function of bamUtil (https://github.com/statgen/bamUtil). Standard quality filters (mapping quality ≥ q25 and base quality ≥ Q30) were applied through samtools version v1.12 *mpileup* function [24]. Reads were deduplicated using MarkDuplicates from Picard. Pseudohaploid variant calling using the Twist Bioscience "Twist Ancient DNA" SNP panel [8] was performed using pileupCaller (https://github.com/stschiff/sequenceTools).

Ancient DNA authenticity, endogenous DNA percentage, fragment size distribution, and post-mortem damage rate at the read termini were determined using DamageProfiler [25].

Mitochondrial data processing

Raw data were processed with the aDNA analysis workflow package nf-core/eager version 2.4.6 [23]. Merged reads with a length greater than or equal to 30 nt were mapped to the mitochondrial revised Cambridge Reference Sequence (rCRS) using CircularMapper (https://github.com/apeltzer/CircularMapper) and *bwa aln* with parameters *-l 1024 -n 0.01 -o 2 -k 2* [26]. Read trimming and filtering followed the procedures outlined above. The read pileups were visually inspected in Geneious v2022.1.1 (Biomatters; https://www.geneious.com). Mitochondrial contamination estimates were calculated using mitoverse HaploCheck version 1.3.2 [27].

Assessment of enrichment efficacy

We note that the word "endogenous" in the term "endogenous DNA content" is loosely interpreted in the paleogenomic literature and often refers to different quantifications. Here, we consider endogenous DNA as the DNA mapping to the reference genome of interest, irrespective of whether that DNA originates to the sample or to contaminant sources. Hereafter, we refer to endogenous DNA percentage as "endo%." We propose the following nomenclature for different calculations of endo%, regarding the specific read counts in the calculation, and whether the calculation is applied to shotgun sequencing data or enriched data. Importantly, we distinguish "endo%," referring to the state of DNA found in the sample, from "post-enrichment endo%" referring to the enriched DNA from the taxon of interest after in-solution target enrichment. For both of these, we define calculations for the sequenced, mappable, filtered, and unique endo% as each informs differently about the usability of the sample and sequenced data. See Tables 1 and 2. for further information.

Cost–benefit analyses

We calculated the financial cost of the experiment on a per SNP basis using the equation below, which captures the combined costs incurred by enrichment and DNA sequencing. The relative difference between the cost of enrichment and the cost of sequencing is expected to impact the economic benefits of enrichment on a case-by-case basis, depending on laboratory-specific commercial agreements for reagents purchase and sequencing service provision, and the sequencing effort.

$$CostOfSNP = \frac{\frac{(CostOfEnrichment * EnrichmentRounds)}{NumberOfSamplesInPool} + \frac{TotalCostOfSequencingRun}{TotalNumberOfReadsSequenced}}{SNPsCovered}$$

Additionally, we computed a generalized linear regression model (for non-normally distributed data), to test the statistical significance of cost saving between TW1 and TW2 compared to shotgun. This was done in R using the formula: $glm(CostOfSNP \sim method + mappable\_endo)$. The mappable endogenous term was added to the model to account for the effect of library endo % on cost-effectiveness.

Allelic bias

Allelic bias was measured using f_4 statistics calculated using *qpDstat* v980 in f_4 mode with options *f4 mode: YES* and *inbreed: YES* in AdmixTools v. 7.0.2 [1].

We performed f_4 statistics of the form f_4 (Mbuti, Pop1.MethodA; Pop2.MethodA, Pop2.MethodB), where the populations were one of East Iberia, West Iberia, Central America, or Southeast Asia and the methods were one of shotgun, TW1, or TW2. In this test, the null hypothesis is that $f_4 = 0$ because the same population (Pop2) is present in the right hand pairing of the equation, but with data deriving from different methods. Therefore, significant deviations from $f_4 = 0$ will indicate an allelic bias deriving from one of the methods.

Index hopping

To estimate single index hopping, we demultiplexed all possible index combinations of the double-indexed libraries. Reads that resulted from the combination of indices initially assigned to two different samples were considered to be the result of index hopping. Subsequently, the rate of single index hopping was determined for every pairwise combination of samples, expressed as the ratio of hopped reads to the sum of hopped reads and all retrievable reads from a sample pair.

For libraries enriched in pools, separate contamination estimates were obtained for reads mapping to the Y chromosome, autosomes, and mitochondrial genome and were used to estimate the impact of double index hopping. We used Haplocheck v1.3.2 [27] to estimate mitochondrial contamination for all the sequenced libraries. The genetic sex of the samples was determined based on the results of SexDetERRmine (https://github.com/nf-core/modules/tree/master/modules/nf-core/sexdeterrmine) from the deep shotgun sequencing data. For all the male samples, we used HapConX [28], an aDNA contamination estimation tool that works using a haplotype copying framework for male X chromosomes to estimate cross-contamination between samples, which in this case is equivalent to double index hopping. Similarly, we calculated contamination estimates using ANGSD [29, 30] for all samples, including the females as a positive control.

Finally, we ran DICE [31], a Bayesian method to estimate the rate of contamination from a specified genetic background, in two-population mode to calculate nuclear contamination for the Central American sample in the pool of four, the three other samples being of Iberian origin. The contaminating population was set to Iberia (IBS) and the anchor population was set to an appropriate Central American population from the 1000 Genomes dataset. The program was run with a Markov chain of 1 million steps and the contamination estimate was restricted to 100 nt either side of each targeted 1240k SNP. We tried to run DICE for Southeast Asian samples with the KHV population from the 1000 Genomes dataset as the anchor. However, the combination of high genetic diversity and underrepresentation of Southeast Asians in the 1000 Genomes dataset meant that we were unable to obtain cross-contamination estimates for our Southeast Asian samples using DICE.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13059-025-03622-6.

Additional file 1. Supplementary figures. Fig. S1 Comparative measures of library quality for shotgun screening data against deep shotgun sequencing. Fig. S2 Effect of pooling on SNPs obtained. Fig. S3 Pairwise rate of single index hopping between every pair of libraries, categorized into 2-library pools TW1, 2-library pools TW2, 4-library pools TW1, 4-library pools TW2, and all other pairs of libraries. Fig. S4 Near-zero contamination estimate suggests minimal index hopping in pooled enrichments.

Additional file 2. Supplementary tables. Table S1 Sequencing statistics and sample metadata including calculated endogenous percentages and cost values. Table S2 Results of genetic sex determination. Table S3 Results of contamination estimates from ANGSD, HapConX, and MT Haplocheck used to infer the impact of index hopping in pooled samples. Table S4 Results of generalized linear model showing that TW2 is statistically significantly more cost-effective than shotgun sequencing. Results of t-tests showing that TW1 and TW2 are significantly more effective than SG. Table S5 Results of the f4 statistical tests presented in Fig. 5.

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Peer review information

Andrew Cosgrove was the primary editor of this article and managed its editorial process and peer review in collaboration with the rest of the editorial team. The peer-review history is available in the online version of this article.

Authors' contributions

R.D., X.R-R., B.L., M.P.W. and L.F-S. conceptualised the study. M.I.M., E.N.I.D., C.S., A.M., L.R.M., A.M.S., S.T., V.M., J.C.T., P.C.C., A.F.M. and T.F. provided samples and R.T. and B.L. funded the empirical research. X.R-R., L.T., C.H., L.F-S., P.W., J.C.T., M.P.W. and E.C. performed laboratory work. Sequence data was processed by S.R., L.T. and analysed by S.R., L.T., R.D. and X.R-R. The manuscript was written by L.T., X.R-R., R.D., B.L., S.R. and R.T. and edited and reviewed by all authors.

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Data availability

The sequencing statistics and cost-benefit datasets generated and analysed during the current study are available in the GitHub repository associated to the publication [32], https://github.com/roberta-davidson/Davidson_etal_2024-Twist/ and is deposited in FigShare [33] with CC-BY 4.0 license (https://doi.org/10.6084/m9.figshare.28968950). The genotype datasets generated and analysed during the current study are made publicly available with de-identified IDs to allow replication of the study [34] (https://doi.org/10.6084/m9.figshare.28733300.v1)

Declarations

Ethics approval and consent to participate

Ethical clearance for the analyses of archeological human remains from Indonesia has been approved by both the National Research and Innovation Agency (BRIN) Ethical Committee in Indonesia (Ethical clearance no: 486/KE.01/SK/10/2022) and the University of Adelaide Human Research Ethics Committee in Australia (Ethics Approval no: H-2020–211). The sampling of the archeological human remains from Mexico was made after approval by the Archaeology Council of the Instituto Nacional de Antropología e Historia with permit number 401.35.16–2018/642. The handling of archeological human remains from Spain was authorized by the housing institution under the framework of Spanish Historical Heritage Law 16/1985.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Australian Centre for Ancient DNA, The Environment Institute, School of Biological Sciences, The University of Adelaide, Adelaide, SA, Australia. [2]Faculty of Arts and Humanities, University of Coimbra, Coimbra, Portugal. [3]Australian Research Council Centre of Excellence for Australian Biodiversity and Heritage (CABAH), School of Biological Sciences, The University of Adelaide, Adelaide, SA, Australia. [4]Mochtar Riady Institute for Nanotechnology, Universitas Pelita Harapan, Tangerang, Indonesia. [5]Laboratory of Genetic Identification & Human Rights (LABIGEN-UGR), Department of Legal Medicine, Faculty of Medicine, University of Granada, Av. Investigación 11, PTS, Granada 18016, Spain. [6]Technology Advancement Unit, Genetics and Molecular Pathology, SA Pathology, Adelaide, SA, Australia. [7]Department of Biology, The Pennsylvania State University, University Park, PA 16802, USA. [8]Research Center for Prehistoric and Historic Archaeology, National Research and Innovation Agency (BRIN), Jakarta, Indonesia. [9]Grup de Recerca en Antropologia Biològica (GREAB), Unitat d'Antropologia Biològica, Universitat Autònoma de Barcelona, Barcelona, Spain. [10]Instituto de Investigaciones Antropológicas, Universidad Nacional Autónoma de México, Mexico City, Mexico. [11]CIAS, Research Centre for Anthropology and Health, Department of Life Sciences, University of Coimbra, Coimbra, Portugal. [12]Department of Life Sciences, CEF, University of Coimbra, Coimbra, Portugal. [13]Centre for Archaeology, University of Lisbon, Lisbon, Portugal. [14]Faculdade de Ciências Sociais E Humanas, Instituto de Estudios Medievais (IEM), Universidade NOVA de Lisboa, Lisbon, Portugal. [15]Department of Biology, University of Évora, Évora, Portugal. [16]Centre for Interdisciplinary Studies (CEIS20), University of Coimbra, Coimbra, Portugal. [17]HERCULES Laboratory and IN2PAST, University of Évora, Évora, Portugal. [18]Evolution of Cultural Diversity Initiative, School of Culture, History and Language, The Australian National University, Canberra, ACT, Australia. [19]Australian Research Council Centre of Excellence for Indigenous and Environmental Histories and Futures, Australian National University, Canberra, ACT, Australia. [20]Department of Anthropology, UCSC Paleogenomics, University of California, Santa Cruz, Santa Cruz, USA. [21]UCSC Genomics Institute, University of California, Santa Cruz, Santa Cruz, USA. [22]National Centre for Indigenous Genomics, John Curtin School of Medical Research, Australian National University, Australian Capital Territory, Canberra, Australia. [23]Indigenous Genomics, Telethon Kids Institute, Adelaide, South Australia, Australia.

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