Allelic bias when performing in-solution enrichment of ancient human DNA

Authors: Roberta Davidson[1#], Matthew P. Williams[1,2], Xavier Roca-Rada[1], Kalina Kassadjikova[3], Raymond Tobler[1,4,5], Lars Fehren-Schmitz[3,6#^], Bastien Llamas[1,5,7,8#^]

Corresponding authors: roberta.davidson@adelaide.edu.au, bastien.llamas@adelaide.edu.au, lfehrens@ucsc.edu

^ Equal contributor

Affiliations

1. The Australian Centre for Ancient DNA and the Environment Institute, School of Biological Sciences, The University of Adelaide, Adelaide, SA 5005, Australia
2. Biology Department, The Pennsylvania State University, University Park, Pennsylvania, USA
3. UCSC Paleogenomics, Department of Anthropology, University of California, Santa Cruz, California, USA
4. Evolution of Cultural Diversity Initiative, Australian National University, Canberra, ACT 2601, Australia
5. Centre of Excellence for Australian Biodiversity and Heritage, The University of Adelaide, Adelaide, SA 5005, Australia
6. UCSC Genomics Institute, University of California, Santa Cruz, California, USA
7. National Centre for Indigenous Genomics, Australian National University, Canberra, ACT 0200, Australia
8. Indigenous Genomics, Telethon Kids Institute, Adelaide, SA 5000, Australia

Abstract

In-solution hybridisation enrichment of genetic variation is a valuable methodology in human paleogenomics. It allows enrichment of endogenous DNA by targeting genetic markers that are comparable between sequencing libraries. Many studies have used the 1240k reagent—which enriches 1,237,207 genome-wide SNPs—since 2015, though access was restricted. In 2021, Twist Biosciences and Daicel Arbor Biosciences independently released commercial kits that enabled all researchers to perform enrichments for the same 1240k SNPs. We used the Daicel Arbor Biosciences Prime Plus kit to enrich 268 ancient samples from four continents. We identified a systematic assay bias that increases genetic similarity between enriched samples and that cannot be explained by batch effects. We present the impact of the bias on population genetics inferences (e.g., Principal Components Analysis, f-statistics) and genetic relatedness (READ). We compare the Prime Plus bias to that previously reported of the legacy 1240k enrichment assay. In f-statistics, we find that all Prime-Plus-generated data exhibit artefactual excess shared drift, such that within-continent relationships cannot be correctly determined. In PCA, the first PCs may capture the assay bias rather than the underlying genetic structure. The bias is more subtle in READ, though interpretation of the results can still be misleading in specific contexts. We expect the bias may affect analyses we have not yet tested. Our observations support previously reported concerns for the integration of different data types in paleogenomics. We also caution that technological solutions to generate 1240k data necessitate a thorough validation process before their adoption in the paleogenomic community.

Keywords

Bias, enrichment, 1240k, ancient DNA

INTRODUCTION

One of the major challenges faced when working with ancient DNA is the high proportion of exogenous DNA contamination present in the DNA extract. This contamination is mostly from microbes that invade the organism post-mortem or are present in the soil where the specimen was buried. It can also be introduced during laboratory experiments. To address this challenge, one method that has become popular in paleogenomic studies is the in-solution enrichment of target genomic regions using pre-designed oligonucleotides as molecular “probes” or “baits”. This technique increases the proportion of endogenous DNA of interest in a sequencing library, reducing sequencing cost and leading to larger quantities of comparable data across individual samples compared to using shotgun sequencing. Ultimately, the increase in analytical power resulting from target enrichment has enabled large-scale ancient human population genetic (i.e., paleogenomic) studies (Fumagalli, 2013).

In human paleogenomic studies, in-solution enrichment assays can be designed to target complete mitochondrial genomes (Briggs et al., 2009; Brotherton et al., 2013; Llamas et al., 2016; Maricic et al., 2010), the non-recombining portion of chromosome Y (Cruz-Dávalos

et al., 2018; Rohrlach et al., 2021), genome-wide SNPs (Fu et al., 2015; Haak et al., 2015; Mathieson et al., 2015), exomes (Castellano et al., 2014), autosomes (Fu et al., 2013), or complete genomes (Ávila-Arcos et al., 2015; Carpenter et al., 2013). In 2012, Patterson and colleagues proposed a molecular bait design that used a specific ascertainment strategy to enable unbiased population genetics studies of global human populations through time (Patterson et al., 2012). The first baits using this design were applied in a handful of landmark paleogenomic studies of European populations (Fu et al., 2015; Haak et al., 2015; Mathieson et al., 2015). These bait sets were subsequently restricted to 1240k genome-wide SNPs, which paved the way for the generation of thousands of individual datasets and launched the paleogenomic era (Marciniak & Perry, 2017; Olalde & Posth, 2020; Skoglund & Mathieson, 2018). Hereafter, we will refer to this set of baits as the 'legacy 1240k reagent'.

Since the initial publication of the molecular baits sequences in 2015, the legacy 1240k reagent was only available to a handful of laboratories through a commercial agreement. Subsequently, in 2021 two biotechnology companies—Daicel Arbor Biosciences and Twist Bioscience—released commercial in-solution enrichment kits that target the same set of 1240k SNPs (Rohland et al., 2022). In particular, the release of the suite of myBaits Expert Human Affinities kits by Daicel Arbor Biosciences aimed to make the 1240k enrichment assay accessible to all research groups worldwide, including those with limited human resources and funding. Importantly, the molecular design of both the new bait sets differs

from the legacy 1240k design, with the differences specific to the Twist reagents described in a recently published study (Rohland et al., 2022). While the bait design used in the myBaits Expert Human Affinities kits is also known to differ from both the legacy 1240k and Twist assays (Daicel Arbor Biosciences, personal communication), it is a proprietary product and the specific details are unknown to the present authors. The bait design and associated laboratory protocol steps of any enrichment reagent are key to its efficacy to enrich endogenous DNA and to achieve an unbiased enrichment that respects the true proportions of reference and alternate alleles at heterozygous sites.

Here, we report genomic data from 268 ancient human individuals from western Eurasia, South and Central America (from periods preceding the colonial contact period between these populations), which were generated using myBaits Expert Human Affinities Kit "Prime Plus" (Daicel Arbor Biosciences) for various independent studies. In addition to the standard set of 1240k SNPs, the Prime Plus (PP) probes target 46,218 Y Chromosome SNPs, as well as human, Neanderthal and Denisovan whole mitochondrial genomes. Notably, when performing routine population genetic analyses on the PP-generated dataset using f-statistics (Patterson et al., 2012), we observed a significant excess of shared genetic drift between any two populations enriched with the PP bait set, compared with published data generated with the legacy 1240k reagent or shotgun sequencing. These unexpected affinities could not be explained by batch effects or previously reported demographic events.

In the following sections, we outline our investigation and characterisation of the bias observed in PP-enriched ancient DNA data via principal components analyses, genetic relatedness analyses and f-statistics. In total, we attempted 21 strategies to reduce or negate the observed bias, which included two wet-lab protocol adjustments and numerous bioinformatic techniques, but we were unable to correct the f-statistics scores to a satisfactory standard. We conclude by recommending a series of principles for researchers to apply when co-analysing PP-enriched data with 1240k legacy and shotgun (SG) data, which mitigate the assay bias by adjusting the construction of f-statistics. These guidelines may also be useful for reviewers and general readers when assessing the robustness of studies that have used data generated with the myBaits Expert Human Affinities Kit "Prime Plus" kit.

MATERIALS AND METHODS

The data used in this study were initially generated for various studies intended by the authors. However, we collated all independent datasets into one overarching dataset after identifying some problems with Prime Plus data, resulting in the present study.

Samples

We generated PP-enriched data for a total of 268 ancient samples, sourced from Spain, Portugal, South-Western Asia, Mexico, Guatemala, Peru, Bolivia, Argentina and Chile. All samples from the Americas pre-date 16th-century European contact (see Table S2 for

130 sample metadata). In our analyses, we use a comparative dataset of 106 shotgun data
131 samples and 291 legacy 1240k samples (Bergström et al., 2020; Bongers et al., 2020; de la
132 Fuente et al., 2018; González-Fortes et al., 2019; Günther et al., 2015; Lindo et al., 2018;
133 Lipson et al., 2017; Mallick et al., 2016; Martiniano et al., 2017; Mathieson et al., 2015;
134 Moreno-Mayar et al., 2018; Nakatsuka, Lazaridis, et al., 2020; Nakatsuka, Luisi, et al.,
135 2020; Olalde et al., 2018, 2019; Popović et al., 2021; Posth et al., 2018; Prüfer et al., 2014;
136 Raghavan et al., 2015; Valdiosera et al., 2018; Villalba-Mouco et al., 2019). A complete list
137 of the comparative samples used in this study and relevant citations are provided in Table
138 S3.
139
140 **Data generation**
141 *DNA extraction*
142 For the samples from Spain, DNA was extracted from tooth root powder using a method
143 optimised to retrieve highly degraded ancient DNA fragments (Dabney et al., 2013) at the
144 Autonomous University of Barcelona (UAB). For Southwest Asian, Argentinian,
145 Portuguese and some Mexican samples, the same methods were undertaken at the
146 Australian Centre for Ancient DNA (ACAD) at the University of Adelaide. For the rest of
147 the samples from the Americas, DNA was extracted similarly, but with a modification to
148 the protocol that includes a bleach pre-wash step (Boessenkool et al., 2017; Dabney et al.,
149 2013), at the University of California, Santa Cruz (UCSC) Human Paleogenomics Lab.
150 Table S2 provides a full summary of which lab processed which samples at each step.

151

152 *Library preparation*

153 For the Southwest Asian, Spanish, Portuguese, Argentinian and some Mexican samples,
154 partially UDG-treated double-stranded DNA libraries (Rohland et al., 2015) were
155 generated at ACAD. For the Bolivian, Peruvian, Chilean, Guatemalan and the rest of the
156 Mexican samples, partially UDG-treated single-stranded DNA libraries (Kapp et al., 2021)
157 were prepared at UCSC. Note that the difference in library structures should not impact
158 the enrichment bias as libraries are first denatured and then hybridised to the baits.

159

160 *Library enrichment*

161 Before enrichment, libraries were over-amplified in order to reach the minimum required
162 DNA input of 500 ng per sample. Library enrichment was then performed according to
163 the suggested myBaits protocol of two enrichment rounds with hybridisation temperature
164 at 70°C and 20 amplification cycles in the first enrichment followed by 10 cycles in the
165 second round. Samples were grouped in pools of 2–4 libraries according to the endogenous
166 DNA proportions obtained from screening of the initial shotgun sequences. Samples were
167 processed at UCSC or at ACAD (see Table S2 for a breakdown of which lab processed
168 which samples at each step).

169

170 Additionally, two experiments that examined the utility of adjustments to standard
171 enrichment protocols were performed on a subset of libraries at UCSC (Table 1). In

experiment A, a single round of hybridisation at 70°C was performed with 12 cycles of amplification. A single round of hybridisation was also used in Experiment B but at 62.5°C and with 13 cycles of amplification.

Sequencing

Libraries enriched at UCSC were paired-end sequenced on a HiSeq 4000 at Fulgent Genomics (Los Angeles, USA). Paired-end sequencing using an Illumina NovaSeq 6000 (200 cycles) was performed by the Kinghorn Centre for Clinical Genomics (Sydney, Australia) for libraries enriched at ACAD (Table S2).

Data processing and genotype calling

Data were processed either using nf-core/eager (Fellows Yates et al., 2021), or UCSC's in-house processing pipeline, Batpipe (https://github.com/mjobin/batpipe). The terminal 2bp at either end of each read were trimmed to remove ancient DNA damage and then mapped to the GRCh37d5 reference genome using bwa-aln with parameters -l 1024 -n 0.01 -o 2 (Oliva et al., 2021). Other mapping parameters vary slightly between different datasets used in this study, such as mapping quality threshold 20-30 and minimum read length 20-30 (see Table S2 for details of processing parameters for each population). Pseudo-haploid genotypes were called at the enrichment target sites using the random haploid mode within pileupCaller (https://github.com/stschiff/sequenceTools).

Population genomics assessment of capture bias

PCA

We computed principal components analyses using both EMU (Meisner et al., 2021) and smartPCA (Patterson et al., 2006; Price et al., 2006) with and without projection to test how the data bias affected each type of PCA.

f-statistics

*O*utgroup-f_3 and f_4 statistics were computed using the ADMIXTOOLS package (Patterson et al., 2012).

READ

We tested the impact of the enrichment bias on genetic relatedness using READ, i.e. Relatedness Estimation for Ancient DNA (Monroy Kuhn et al., 2018), which is a widely used PMR-based estimator for genetic relatedness for ancient DNA data.

Filters and strategies to resolve bias

In order to estimate the effect of various bioinformatic approaches to mitigate the bias observed in f_4 statistics, we calculated f-statistics in the following four forms:

1) f_4(Mbuti, Spain.PP.2rnd; Target, Peru.Shotgun)
2) f_4(Mbuti, Spain.PP.2rnd; Target, Peru.1240k)
3) f_4(Mbuti, Spain.1240k; Target, Peru.Shotgun)
4) f_4(Mbuti, Spain.1240k; Target, Peru.1240k)

215 In each, the target population was the Peru.PP population after application of one of the
216 21 strategies to reduce bias detailed below.
217
218 *SNP Missingness*
219 Amongst the set of 1240k SNPs assayed by the PP probes, each SNP was missing at least
220 once in our dataset. Hence, we used the full data matrix with missing genotypes and
221 reported the number of overlapping SNPs included in each f-statistic comparison where
222 relevant.
223 To the target population (Peru.PP), we applied two data missingness filters using the --
224 *geno* flag in PLINK 1.9 (Chang et al., 2015)—namely, 0.0 and 0.5, which correspond to
225 removing SNPs with >0% or >50% missingness, respectively.
226
227 *Minor allele frequency*
228 We tested some simple filters for minor allele frequency (i.e., 0.3, 0.1 and 0.01) using the -
229 -*maf* flag in PLINK 1.9 (Chang et al., 2015).
230
231 *Linkage disequilibrium*
232 We filtered for linkage disequilibrium (LD), with the intention to remove potential
233 artefactual linkage introduced by one bait targeting multiple SNP sites. We used the
234 PLINK 1.9 LD pruning parameter --*indep-pairwise* with a window size of 3 variants,
235 sliding in 1 variant steps and tested correlation threshold values of r^2 = 0.1, 0.3, 0.5, 0.7

236 and 0.9 (Chang et al., 2015). These filters resulted in datasets of 300,838, 346,428, 379,345,
237 388,038, and 388,678 SNPs, respectively.
238
239 Given that each r^2 threshold produced approximately 300,000 SNPs, to assess if the
240 improvement in f_4-value was driven by the reduction in bias attributable to artefactual
241 linkage, rather than changes to other statistical properties of the data (such as decreased
242 power due to a reduced number of SNPs), we repeated our analyses with random sub-
243 samples of 300,000 SNPs, replicated five times, using the PLINK 1.9 flag *--thin-count*
244 *(Chang et al., 2015)*.
245
246 *Transversions*
247 Sequencing libraries were partially treated with UDG (Kapp et al., 2021) and the terminal
248 two nucleotides at either end of all mapped reads were trimmed when processing the data
249 with nf-core/eager (Fellows Yates et al., 2021). Therefore, we did not expect to observe a
250 strong impact of ancient DNA damage in the analyses. Nevertheless, we filtered out
251 transitions to minimise the potential impact of ancient DNA damage, which is typically
252 characterised by C-to-T substitutions at the 5' end of sequencing reads and C-to-T or G-
253 to-A substitutions at the 3' end of sequencing reads when using single-stranded or double-
254 stranded libraries, respectively (Krause et al., 2010; Meyer et al., 2012).
255
256 *Application of Rohland et al. 2022 filter*

257 We applied the filter suggested by Rohland and colleagues (Rohland et al., 2022) to extract
258 SNPs that will allow co-analysis between 1240K data generated with shotgun sequencing,
259 or the legacy 1240k, Prime Plus or Twist enrichments.
260
261 *PCA derived filter*
262 PCAs computed for a combination of PP-, legacy-1240k- and shotgun-generated ancient
263 samples with smartPCA (Patterson et al., 2006; Price et al., 2006) displayed patterns
264 consistent with assay bias along PC2. Hence, we used this principal component to
265 construct an assay-based filter by extracting SNPs weighted > |1| in PC2 of Fig 2B (n =
266 129,982 SNPs). The SNP weight threshold was validated by the observed removal of the
267 assay bias along PC2 when computing PCA after applying this filter (Fig 2C).
268
269 *Read length and mapping quality threshold*
270 We filtered out reads that were shorter than 50 bp and/or with mapping quality less than
271 20, as suggested by Günther & Nettelblad to reduce reference bias when mapping
272 paleogenomic data (Günther & Nettelblad, 2019).
273
274 *Off-target genotypes*
275 Given the hypothesis that the bias is caused by bait-target interactions and that the
276 enriched library efficacy is not 100%, we called off-target (non-specific) genotypes on the
277 assumption that they would not be impacted by any bait-mediated mechanism driving the

278 bias. The genotyping of off-target sites was performed using a standard pseudo-haploid
279 pileupCaller call with the dbSNP set 138 (Sherry et al., 2001) after filtering out all PP
280 target sites. Additionally, we called genotypes that were at least 100 bp away from target
281 sites to eliminate SNPs that may have been covered by a bait but were not the target
282 locus.
283
284 *Majority call pseudo-haploid and random diploid genotypes*
285 In addition to standard pseudo-haploid calls based on the random selection of a single
286 allele at prespecified SNPs, majority allele pseudo-haploid genotypes were also called at
287 sites with greater than 5X depth-of-coverage using the majority call mode in pileupCaller.
288 Finally, diploid genotypes were called using the random diploid call mode in pileupCaller,
289 where one read is chosen at random, followed by a second chosen at random without
290 replacement, and sites covered by only one read are considered missing.
291
292 **Investigation of allele call bias**
293 We investigated the rate of each allele call at heterozygous SNP sites for the most
294 common substitutions: (A/C, T/G, A/G, T/C), where the inverse substitution was also
295 included such that i.e., A/C and C/A SNPs are collated together. The relative allele call
296 frequencies were calculated per SNP (ignoring missing SNPs) in aggregate across all
297 population datasets for shotgun-, legacy-1240k- and PP-enriched genotypes. SNP

missingness was quantified across 100 bins with equal numbers of SNPs, and the mean allele frequency per bin was plotted against SNP missingness.

RESULTS

Global bias assessment

To quantify the extent of the PP-associated bias in outgroup-f_3 statistics, we calculated statistics of the form f_3(Mbuti; Target, X), where Target refers to samples that were enriched using PP and originate either from ancient Spain (Spain.PP.2rnd; unpublished) or Peru (Peru.PP.2rnd; unpublished), and X denotes a set of South American and European ancient reference populations either generated from shotgun sequencing or enriched with the PP or legacy 1240k kits.

For f_3 statistics that use Spain.PP.2rnd as the Target population (Fig 1A), all non-PP European populations (shotgun or legacy 1240k data) exhibit more shared drift with the Spain.PP.2rnd population than do the South American populations of either enrichment type (Fig 1A), and the relative relationships between the European populations are consistent with previous reports (Martiniano et al., 2017; Olalde et al., 2019; Valdiosera et al., 2018). However, the four South American PP populations share more genetic drift with the Spain.PP.2rnd target population relative to other non-PP South American populations. Similarly, the inter-continental relationship is preserved when Peru.PP.2rnd is used as the target population, as all South American populations have substantially more

shared drift with the Peru.PP.2rnd target population than with European populations, yet the intra-continental relationships appear to be violated by the biased shared drift between PP populations (Fig 1B). Further, Spain.PP.2rnd and Portugal.PP.2rnd exhibit a strong attraction toward Peru.PP.2rnd samples relative to the other European non-PP populations.

We also observe variance between 1240k and shotgun datasets, which is likely due to a combination of different ancestries and 1240k bias (Rohland et al., 2022), however in most cases the shotgun and 1240k error bars overlap, whereas the Prime Plus bias is more pronounced. Clearly, the bias observed in PP-generated data is not unique to South American populations but is ubiquitous across the highly diverged populations of Europe and South America, strongly suggesting that the bias affects all global populations.

Principal Components Analysis

We tested the impact of PP bias on multiple PCA approaches, including the widely used smartPCA (Patterson et al., 2006; Price et al., 2006) and EMU, which is reported to handle data missingness well (Meisner et al., 2021). For each approach, we computed PCAs at continental and global scales to investigate how missingness and the PP bias manifested at different geographical scales.

Continental PCA

340 Initially, we performed a PCA using EMU on a dataset of 386 ancient genomes from South
341 America including shotgun (n = 38), legacy 1240k (n = 223) and PP-enriched (n = 125)
342 data (see Table S1 for a detailed list of samples). Sample clustering in the first three
343 principal components of the PCA is driven by population structure (Fig S1, right) and
344 there is no apparent bias exhibited by the data source type (Fig S1, left). Note that no
345 individual samples were assayed with each of the three methods investigated, but many
346 samples of different data types derive from the same localised ancestry groups. This result
347 concurs with a recent study (Rohland et al., 2022), which also reported that PCA is robust
348 to combinations of these three data types. However, when we used smartPCA on the same
349 386 ancient genomes (without projection onto modern diversity), the resulting PCA
350 produced sample clustering along PC2 and PC3 that was driven by the assay type rather
351 than ancestry (Fig S2). Additionally, samples moved toward the origin due to SNP
352 missingness (Fig S4), an issue known to affect smartPCA that is largely circumvented by
353 EMU in the present study.
354
355 *Global PCA*
356 Subsequently, we performed another PCA with EMU that included the 386 ancient South
357 American genomes described above, and an additional 107 ancient Iberian individuals
358 from Spain, Portugal and Gibraltar of the three investigated data types (Table S1). While
359 PC1 is driven by the ancestral difference between South American and Iberian

360 populations, as expected, PC2 is driven by the assay type and PC3 again represents
361 ancestry according to expected relationships (Fig 2A).
362
363 We then repeated this PCA but using smartPCA with outlier removal and no projection
364 (Fig 2B). While this PCA looks significantly different there is still a clear separation by
365 continent along PC1, assay along PC2 and by South American regional ancestry along
366 PC3. This strongly suggests that the allele frequency variation driving PC2 comes from
367 SNPs that are strongly affected by the assay bias, at least for this dataset. Hence, we
368 leveraged the SNP loadings outputted by smartPCA to construct a PCA-based filter that
369 removed all SNPs weighted > |1| from the dataset (n = 129,982 SNPs). After applying this
370 filter to the data and recomputing the PCA (Fig 2C), the 'assay component' is no longer
371 evident along PC2—which instead captures South American regional variation, as did
372 PC3 before the filtering (Fig 2B) — whereas PC3 is driven by variation among just a few
373 individuals. Lastly, we made use of the smartPCA projection functionality and projected
374 the PP samples onto the PCA generated from the non-PP samples (Fig 2D). This PCA
375 looks strikingly similar to the PCA constructed from the filtered data in Fig 2C.
376
377 *Global PCA with smartPCA projection*
378 To assess the effect of the enrichment bias on a global scale, we applied both EMU and
379 smartPCA projection methods to 612 ancient genomes from South and Central America,
380 Europe and Southwest Asia, either generated by shotgun sequencing or enriched using the

381 PP or legacy 1240k kits, onto a PCA of modern diversity that used 1,071 individuals from
382 the HGDP dataset (Bergström et al., 2020) (Fig 3).
383 When using EMU, the ancient individuals are projected with similar modern ancestries
384 (Fig 3, left). However when using smartPCA (Fig 3, right), the ancient samples are
385 projected into the correct quadrant of the PCA, i.e., clustering within the correct
386 continent, but data missingness seems to drive other discernible patterns, as both the
387 ancient American and Eurasian samples form clines from the plot origin to their
388 respective modern sample clusters located at either end of PC2. While PP samples are
389 situated closer to the PCA origin than the legacy 1240k samples, this can be explained by
390 the higher rate of data missingness in the PP dataset (see Fig S4).
391
392 **Genetic relatedness**
393 We ran READ for pairwise combinations of four individuals from Peru for which we had
394 generated shotgun data and single round PP-enriched data as a part of Experiment B (see
395 Methods). These individuals represent the only samples for which both shotgun data and
396 PP-enriched data were available. Given that only a single round of enrichment was used
397 in these PP assays, technical bias is expected to be reduced relative to double-round
398 enrichments.
399 The four artefactual "true twins" pairs (i.e. the same individual assayed with different
400 methods; e.g., IndA.PP.1rnd.B and IndA.Shotgun) were the most related of all
401 combinations with all average P0 values <0.4 (Fig S3). The remaining "non-twin" pairs all

402 had a P0 value of close to 0.5, with small increments between them. Notably, PP-enriched
403 pairs (e.g., Ind1.PP.1rnd.B and Ind2.PP.1rnd.B) were always more closely related (lower
404 P0) than the respective shotgun pairing (e.g., Ind1.Shotgun and Ind2.Shotgun) (Fig S3).
405 This suggests that the PP bias effects READ inferences, although it is subtle in this
406 instance.
407
408 **Comparison of strategies to reduce bias**
409 We conducted two wet-lab experiments with altered enrichment protocols and applied a
410 total of 19 bioinformatic strategies, aiming to find a strategy that best reduced the Prime
411 Plus bias. Bioinformatic strategies are categorised according to whether they 1) reduce
412 mapping reference bias, 2) reduce the impact of ancient DNA damage, 3) remove specific
413 "biased" SNPs, 4) employ statistical filters based on allele frequencies or 5) call genotypes
414 with alternate methods. Our test population comprised the ancient Peruvian samples, the
415 only population for which we generated both Prime Plus and shotgun data (Bongers et al.,
416 2020). This allowed us to compare the effect of bias-reduction strategies in an f_4 test
417 using statistics of the form f_4(Mbuti, Spain.PP.2rnd; Target, Peru.Shotgun), where the
418 target rotates between each of the different bias reduction strategies applied to the
419 Peruvian population. We chose Spain.PP.2rnd for this assessment for the following
420 reasons: i) the Spanish dataset was generated in a different lab, by different individuals,
421 and was sequenced on a different sequencing machine, than the Peruvian dataset (Table
422 S2), thereby ruling out batch effects and isolating the Prime Plus enrichment kit as the

underlying cause of any bias; and ii) the Spanish and Peruvian populations are sufficiently divergent and lack evidence of recent admixture (the Peruvian data date to pre-colonial times) such that the impact of Prime Plus bias is readily observable, but is not stronger than the population affinities, as would be the case if we were comparing two more closely related groups (such as populations from the same continent). To quantify the impact of Prime Plus bias relative to population affinity between the Spanish and Peruvian samples, we included Spain.1240k and Spain.Shotgun from publicly available data as target populations.

In total, we calculated f_4 statistics in the following four configurations to robustly estimate the Prime Plus bias and determine the best strategies to reduce it (Fig 4):

1) f_4(Mbuti, Spain.PP.2rnd; Target, Peru.Shotgun)
2) f_4(Mbuti, Spain.PP.2rnd; Target, Peru.1240k)
3) f_4(Mbuti, Spain.1240k; Target, Peru.Shotgun)
4) f_4(Mbuti, Spain.1240k; Target, Peru.1240k)

The reason for using the first two configurations is that by comparing the target populations with Peruvian shotgun data, we are measuring our attempts to resolve the Prime Plus bias against the least biased data currently available. However, this may be an unreasonable standard to meet, given that it is known that legacy 1240k data also has some biases (Rohland et al., 2022), and the bulk of published ancient DNA data is legacy 1240k data. Therefore, while this comparison may not be a perfect estimation

of total bias reduction, it will provide more useful insights for researchers given that most projects will leverage large proportions of 1240k reference data. Following the same logic, in the third and fourth configurations, we also computed f_4 statistics with Spanish legacy 1240k data (Fig 4), to gain a deeper understanding of the merits of alternate strategies for reducing bias in different combinations of PP, legacy 1240k and shotgun data.

As a positive control, we included a different set of ancient shotgun genomes from Peru (Peru.other.Shotgun) as a Target population, which resulted in f_4 results that were not significantly different from zero ($Z < 3$), as expected. The main aim of this process was to identify a strategy that would behave like the positive control, i.e., that would reduce the enrichment bias (Peru.PP.2rnd; dashed vertical line, Fig 4) so the f_4 values are not significantly different from 0. In the first configuration f_4(Mbuti, Spain.PP.2rnd; Target, Peru.Shotgun), a result not statistically different from 0 would indicate the bias was alleviated, and in the second configuration f_4(Mbuti, Spain.PP.2rnd; Target, Peru.1240k), the same result would suggest that the PP data is at least co-analysable with 1240k data under specific configurations. Importantly, the third configuration f_4(Mbuti, Spain.1240k; Peru.PP.2rnd, Peru.Shotgun) (Fig 4) resulted in an f_4 result that was not significantly different from zero, indicating that there was no significant bias between these PP and legacy 1240k datasets. Finally, for the fourth configuration f_4(Mbuti, Spain.1240k; Peru.PP.2rnd, Peru.1240k) (Fig 4) the f_4 result is significantly positive, providing further evidence for the previously reported bias in the

legacy 1240k enrichment (Rohland et al., 2022). Therefore, the purpose of including Spain.1240k among our tested configurations is to find a bias-reduction strategy that does not significantly impact these f_4 statistics, and that allows the confident application of bias reduction strategies to different combinations of the Prime Plus and other data types and under alternate f_4 statistic configurations.

Ancient DNA damage or reference bias

The filter for minimum read length (Peru.PP.2rnd.>50bp) suggested by (Günther & Nettelblad, 2019) to reduce mapping reference bias had minimal effect on the f_4 statistics, suggesting that the observed assay bias is not strongly impacted by very short reads.

Filtering out transition SNPs (A/G and C/T), thus removing any possible ancient DNA damage (Peru.PP.2rnd.transversion) had a negligible effect on the f_4 statistics scores. Indeed, because all libraries were half-UDG treated and the ends of sequencing reads trimmed, we did not anticipate that ancient DNA damage would have a large impact.

SNP-set based filters

Strategies to reduce bias by removing certain SNPs included filtering to include only the Human Origins SNP set (Peru.PP.2rnd.HO; 593,120 SNPs retained), filtering according to the SNPs driving PC2 (the "assay component" in Fig 2B, Peru.PP.PCAfilter; 486,445 SNPs retained), and a filter previously reported (Rohland et al., 2022) to enable the co-analysis

of legacy 1240k and Prime Plus data (Peru.PP.2rnd.Rohland; 803,662 SNPs retained). Retaining only the Human Origins SNPs or applying the PCA- based filter had minimal effect on the f_4 results. Notably, the application of the Rohland filter did not alleviate bias in any of the configurations of the f_4 statistics that we tested. In some cases application of the Rohland filter resulted in the f_4 values shifting slightly toward zero, but in other cases slightly away, which is likely due to an increase in uncertainty from the approximately 2-fold reduction in the number of SNPs. Overall, this indicates the Rohland filter does not work as reported on the present dataset, suggesting that it is not a universally applicable solution for capture bias.

Allele frequency-based filters.

We further examined the bias reduction efficacy of a series of filters based on allele frequencies, including filters for SNP missingness, minor allele frequency and linkage disequilibrium. Of the SNP missingness filters, a strategy that excluded sites missing in >50% of individuals (Peru.PP.2rnd.miss0.5) did not notably shift the f_4 value towards zero. However, excluding all SNPs exhibiting any missingness (Peru.PP.2rnd.miss0) did move the value toward zero, but it also reduced the number of SNPs so drastically that this strategy would probably not be suitable for most paleogenomic studies. For SNP filters based on minor allele frequency (Peru.PP.2rnd.MAF0.3, Peru.PP.2rnd.MAF0.1 and Peru.PP.2rnd.MAF0.01), f_4 results were shifted toward zero as the stringency of the filter increased, but this also led to increasingly large reductions in the number of analysable SNPs. Interestingly, in the

f_4 configurations with Spain.1240k, the minor allele frequency filters produced positive f_4 values indicating a bias towards the Peruvian data regardless of whether shotgun or legacy 1240k are used.

When applying LD-based filters, we identified that the shared drift with Spain.PP.2rnd reduces as the stringency of the filter increases, though this once again came at the cost of increasingly fewer retained SNPs. In the configuration f_4(Mbuti, Spain.PP.2rnd; Target, Peru.Shotgun), the f_4 result for $r^2 = 0.1$ was very close to 0 (Peru.PP.2rnd.LDr0.1). Filters for less stringent r^2 values filtered out fewer SNPs but did not improve f_4 results to the same degree (Fig 4). In the configuration f_4(Mbuti, Spain.PP; Target, Peru.1240k), the less stringent $r^2 = 0.3$ produces an f_4 value not significantly different from 0, with the retention of more SNPs than the $r^2 = 0.1$ filter. The use of this filter to alleviate PP bias could be considered by users in specific cases with high sequencing depth to accommodate the expected SNP loss. Randomly downsampling the Peru.PP data to an equivalent number of SNPs (300,000) yielded f_4 values with additional variance around the value of the unfiltered Peru.PP data (dashed lines, Fig 4) and higher absolute Z-scores (Fig 4). This suggests that the bias may in fact be creating artificial "linkage" among neighbouring SNPs, but that sufficiently stringent LD pruning to "correct" the value to zero requires discarding the majority of targeted SNPs. Accordingly, the value of linkage disequilibrium pruning may be considered as a potential solution to the PP bias on a case-by-case basis, with the caveat that improved bias correction will be offset to some degree by the loss of

precision from reduced statistical power, and consideration will need to be given to the effect of LD pruning on the results of various analyses.

Alternative genotyping

We considered several genotyping approaches alternative to the standard random pseudo-haploidisation, including calling random diploid genotypes, using majority call pseudo-haploid genotypes at sites with > 5X coverage, genotyping off-target SNPs, and genotyping 'out-of-range' SNPs that were > 100 bp from the target sites. Calling off-target genotypes (Peru.PP.2rnd.OffTarget) and out-of-range genotypes (Peru.PP.2rnd.>100bpOffTarget) both resulted in a small shift of the f_4 values toward zero. Majority call genotypes (Peru.PP.2rnd.>5X.maj) behaved very differently between configurations. In configurations with Spain.1240k, the filter had a minimal effect, but with Spain.PP.2rnd, the majority call genotyping drastically shifted the f_4 results towards negative values, indicating that this approach increases the artefactual "shared drift" between PP data from Spain and Peru. Random diploid genotyping produced an f_4 value that was even more biased (Peru.PP.2rnd.diploid), mildly so when compared with Spain.1240k, but much more strongly for Spain.PP.2rnd. In fact, the latter value is close to the f_4 result where the target dataset is Spain.Shotgun. This means that using random diploid genotype calls exacerbates the artefactual shared drift driven by the PP enrichment bias to approximately the same magnitude as true shared drift between Spanish populations (Fig 4).

553 *Laboratory experiments*

554 In our final set of bias reduction strategies, two non-standard lab experiment protocols
555 that used a single round of enrichment (Peru.PP.1rnd.A and Peru.PP.1rnd.B) both led to
556 minor bias reductions. Protocol B, which lowered the hybridisation temperature
557 performed slightly better than Protocol A. While the overall improvement exhibited by
558 both methods was minor, the bias remained considerably greater than the bias observed in
559 the legacy 1240k data (Peru.1240k).

560

561 **Allele calls**

562 We observe strong GC bias amongst the data generated with the legacy 1240k baits,
563 whereas the PP data display comparatively lower levels of bias that are broadly
564 comparable with shotgun datasets (Fig 5). It is important to note that the bias apparent in
565 the legacy 1240k data was not a concern prior to the release of alternate enrichment kits,
566 simply because all the data generated using the legacy 1240k baits are comparable. We
567 observe that across all three methods, the GC bias is stronger for transitions—i.e., allele
568 pairs A/G and C/T where both alleles are either purines or pyrimidines, respectively—
569 which represent the majority of target sites (80%). Though our understanding of the
570 biochemical mechanisms underlying the PP bias is limited by a lack of knowledge of the
571 bait design, our data clearly demonstrate a systematic difference in the pattern of allelic
572 biases between the legacy 1240k and PP assays.

573

DISCUSSION

Results presented in this study support the previous report of a bias when enriching SNPs at ~1240k sites using the Daicel Arbor Biosciences Expert Human Affinities Prime Plus enrichment kit, and extend the previous findings to ancient Indigenous American (i.e., non-Eurasian) populations. In accordance with (Rohland et al., 2022) we find that the biased data strongly affect f-statistic calculations, where the shared genetic drift (i.e., the evolutionary history) between groups is explicitly estimated. Note that we consider the effect strong, not because of the magnitude of any quantitative measurements, but due to the corruption of expected demographic relationships between populations that is driven by this bias. In contrast with results reported by (Rohland et al., 2022), we find that the Prime Plus (PP) bias does affect PCA when PP-enriched samples are included in the PCA calculation (i.e. not projected onto PCs computed from modern genomes), as might be expected given the close relationship between PCA and f-statistics (Peter, 2022). Additionally, for the first time, we observe a (in this case mild) bias effect on the predicted relatedness between individuals using the ancient DNA relatedness test READ.

Batch effects

The initial observation that PP data had an unexpected excess of shared drift with other PP-generated data in f-statistics prompted us to investigate if this artefact was due to a batch effect arising from certain samples, specific labs having done the work, DNA sequencing machines, or resulted from the distinct demographic history of populations in

596 question. We can confidently rule out each of these hypotheses, as the lab work was
597 performed on numerous sample sets, in different labs, by multiple individuals, sequenced
598 in different facilities using different sequencers (Table S2), with the only common factor
599 to all biased samples being the PP enrichment. Our outgroup-f_3 statistics calculated
600 between European and South American populations (Fig 1) confirmed that the bias
601 affected these two highly diverged populations suggesting that it is most likely a
602 systematic bias that affects all human ancestries. However, we did not have access to the
603 appropriate shotgun data to determine if the enrichment bias has differential effects by
604 ancestry source, and this hypothesis requires additional testing.
605
606 ***Describing the Prime Plus bias***
607 *PCA*
608 We performed principal components analysis in several ways with the aim of addressing
609 multiple analytical approaches that researchers might take. To accommodate differing
610 geographical scales, we computed separate PCAs at continental and global scales. While
611 the approach of using smartPCA with projection has long been considered the gold
612 standard, it should be noted that this method works best with high-quality ancient
613 samples that have low missingness and a well-developed reference database of modern
614 diversity that captures the genetic variation present in the ancient populations, therefore
615 enabling the most accurate projection. Hence, the PCA published by Rohland et al. 2022
616 could be expected to show good results as they projected 15 high-quality ancient genomes

617 onto the modern diversity of western Eurasia, the most studied region of the world.
618 However, this is generally less achievable in understudied regions where modern
619 reference data is lacking and/or when working with low-quality samples. This is what
620 motivated the simultaneous use of EMU (designed to handle data missingness) (Meisner et
621 al., 2021) and smartPCA both with and without projection onto modern diversity.
622
623 At the continental scale, EMU produced a PCA that reflects South American population
624 structure in accordance with previous results (Nakatsuka, Lazaridis, et al., 2020;
625 Nakatsuka, Luisi, et al., 2020; Posth et al., 2018) and patterns suggesting assay bias are not
626 evident within the first three principal components (Fig S1). In contrast, PC2 in the
627 smartPCA (without projection) clearly distinguished data generated from the legacy
628 1240k enrichment from PP data (Fig S2), and exhibited patterns reflective of data
629 missingness (i.e., samples with more missingness being drawn toward the origin), which
630 was not apparent in any EMU PCA (Fig S2).
631
632 However, at the global scale, the PP bias was apparent in PCAs produced by both
633 smartPCA and EMU (Fig 2A and 2B, top row), although the resulting patterns look very
634 different. In both cases, the PP and legacy 1240k samples occupy largely distinct regions
635 of PCA space, although this divergence is less pronounced in South America than in
636 Iberia, suggesting that the bias may have a variable effect on different ancestries. Plots of
637 PC1 against PC3 (Fig 2A and 2B, bottom row) then reflect the clustering that we would

expect, separating South America from Iberia along PC1 and then the Andes from the Southern Cone of South America along PC3. It is notable that the smartPCA (without projection) output is still highly affected by missing data whereas this did not obviously affect the EMU results.

The most striking discrepancy between the two PCA methods came from smartPCA's projection of ancient genomes onto the modern diversity of the HGDP data (Bergström et al., 2020). In the smartPCA output (Fig 3, right), while the shotgun genomes are in accordance with previous results, both the legacy 1240k and PP samples are drawn toward the origin, most likely due to missingness in the data (Fig S4). Similarly, the apparent increased clustering of the PP samples relative to the legacy 1240k data may also be driven by the higher rate of missingness amongst the PP data in this study (Fig S4). Ultimately, these underlying issues mean that this PCA is not suitable for making demographic inferences. In contrast, the EMU PCA places all ancient individuals within the proximity of their associated modern population. Note that EMU does not have projection functionality and instead includes all individuals in the calculation of eigenvectors while imputing sites with missing data (Meisner et al., 2021). There does not seem to be any obvious assay bias in the EMU PCA output, although this might still be present in a lower-level PC.

Overall, we suggest that PCAs built with both smartPCA and EMU methods should be produced and compared, especially in cases where PP data, or data with high rates of missingness are included. PCAs are likely to be more robust at a smaller geographical scale, and if computed from ancient samples, will benefit from including a majority of non-PP samples to drive the assay bias component to a lower-level PC, thereby enabling broad interpretations of the first two to three PCs.

Genetic relatedness

We assessed the effect of PP bias on results from the genetic relatedness estimation software READ (Monroy Kuhn et al., 2018). We found that pairwise estimations between PP libraries were always more closely related than shotgun library pairs of the same four samples (Fig S3). Given that these PP libraries were only enriched for a single round, we would expect this bias to be even stronger in PP libraries that follow the standard two-round enrichment protocol (reflecting patterns observed in the f_4 statistics; Fig 4). In the present case, the bias is subtle and unlikely to lead to, for example, a false-positive first-degree relationship from an unrelated pair. While our ability to make more general conclusions is clearly limited by the sample set, our results tentatively suggest that READ inferences should be reasonably robust if run on a dataset of only PP data, but users should exhibit caution when combining different data types.

f-statistics

Our analyses of the PP bias in both outgroup-f_3 and f_4 statistics showed that the bias is strong enough to corrupt expected relationships between populations from the same continent (Fig 1). We reproduced the legacy 1240k bias that has been previously reported (Rohland et al., 2022) and showed that it is relatively subtle compared to the PP bias, indicating that it is less impactful on population genetic inferences.

Importantly, our analyses suggest that it should be possible to use Prime Plus data in a comparative outgroup-f_3 if the target population is the only one enriched with Prime Plus, that is statistics of the form f_3(Outgroup.notPP; target.PP, X.notPP) where both the outgroup and population X are not enriched with Prime Plus. This is suggested by our f_3 statistics (Fig 1A) where Spain.PP.2rnd is the Target population, as the three European populations reproduce the expected relative relationships previously reported (Martiniano et al., 2017; Olalde et al., 2019; Valdiosera et al., 2018) despite none having been enriched with the Prime Plus baits. However, the limitation is that a population enriched with Prime Plus could not be split into subpopulations, such as Spain_A.PP.2rnd and Spain_B.PP.2rnd, and rotated in the X population position, or in scenarios where one is X and the other Test, but then compared to f_3 statistics of a different form. Presumably, an f-statistic would also be viable if all X groups were PP-enriched because the enrichment bias would be shared by all data and should not corrupt demographic interpretations of the results. See Table S4 for a comprehensive list of acceptable outgroup-f_3 and f_4 statistic forms and, though not directly investigated here, using the

same logic we also list qpWave and qpAdm configurations and their relative reliability under PP bias.

Strategies to reduce bias

The bias observed in f-statistics is both persistent and systematic, which allowed us to test a total of 21 experimental and analytical protocols to characterise the bias and alleviate its disruptive impact on inferences from common population genetic analyses. Many of these analytical protocols produced marginal improvements, but no protocol was sufficient to remove all biases (Z-score remained such that $|Z| > 3$, and/or resulted in low SNP retention).

Ancient DNA damage or reference bias

Neither ancient DNA damage nor reference bias appeared to contribute to the bias, as removing transition sites (all SNPs that could potentially be ancient DNA damage) and removing reads shorter than 50 bp had little impact on bias in the f_4 statistics (Fig 4).

SNP-set based filters

Next, we investigated if a subset of the 1240k SNPs was driving the bias and could therefore be removed to allow safe co-analysis of PP enriched samples with other data types. This idea was initially suggested by Rohland and colleagues (Rohland et al., 2022),

who published a list of SNPs that they suggested should be suitable for co-analyses of shotgun, legacy 1240k, Twist, and PP data. However, our tests of the Rohland et al. filter produced no notable improvement on the f_4 statistics when co-analysed with 1240k or PP data (Peru.PP.2rnd.Rohland, Fig 4). Note that to build this filter Rohland and colleagues looked for improved Z-scores, i.e., closer to zero, but did not report the f_4 statistics that are informative to estimate the effect on population relatedness. When using any SNP filter, the removal of SNPs will likely increase the standard error of the f_4 statistic, leading to decreased statistical power and precision. However, it is not possible to predict the deviation from the true f_4 statistic. Therefore Z-scores alone do not serve as a measure of the bias-reduction of a filtering strategy. This emphasises the importance of testing hypotheses in the context of the study and understanding the expected relationships of the populations in question.

Subsetting our data to include only the ~600k SNPs in the Human Origins set had a similar effect to the Rohland filter. This may be related to the overlap between the two sets, 61% of the Human Origins SNPs are also present in the Rohland filter set. (367,332 intersecting SNPs).

Additionally, we generated our own list of SNPs to filter by extracting the SNPs that were driving variation evident in PC2, the “assay component” in the smartPCA output (Fig 2B). Although this filtering approach did seem to remove the biased component from the

742 PCA (Fig 2C), it had a negligible impact on any of the f_4 statistics. In summary, none of
743 these filtering strategies corrected the PP bias satisfactorily. Furthermore, while Rohland
744 et al. acknowledge there is room for improvement in their filtering approach, it is possible
745 that their filter is well-calibrated for the Eurasian ancestries present in their dataset but
746 does not represent a universal filter to mitigate Prime Plus capture bias in all ancestries. In
747 any case, it is less than ideal to have enrichment kits on the market that would require
748 discarding a subset of target SNPs when combining multiple data types. This will
749 negatively affect not only users of the Daicel Arbor Biosciences Human Affinities kits —
750 and potentially other commercial kits — but also impact subsequent analyses by other
751 researchers that incorporate published PP datasets.
752
753 *Alternative genotyping*
754 Genotype calling in paleogenomic studies typically involves randomly sampling one read
755 covering a targeted SNP site, known as 'pseudo-haploidisation', making it possible to
756 generate allele calls at sites with low coverage (Morris et al., 2011). Given that our
757 experiments with SNP filtering did not produce a universal solution and following the
758 assumption that every targeted SNP site may be affected by the bias, we investigated the
759 efficacy of alternate genotype calling for bias reduction. In our first assessment, we called
760 pseudo-haploid genotypes at off-target SNPs, which we hypothesised would be equivalent
761 to shotgun data and therefore unbiased if the bias was driven by the bait design. Similarly,
762 we also called random pseudo-haploid genotypes at sites more than 100 bp away from a

target locus to mitigate the possibility of an "off-target" site being covered by a probe that had correctly hybridised to a nearby target locus. The f_4 statistic for both off-target and out-of-range genotypes did improve compared to the on-target results, producing values that were not significantly different from zero (Fig 4). Interestingly, the f_4 statistic for the out-of-range genotypes was not notably different from the off-target estimation. Given that all non-bound DNA fragments are theoretically washed away during the enrichment protocol, the off-target bait bias is likely due to an as-yet-unknown biochemical bias that systematically affects bait binding to DNA at non-target locations, but it is unlikely this is caused by on-target hybridised baits covering nearby SNPs within a 100 bp range.

Additionally, we used an alternate pseudo-haploid calling approach which requires target SNPs to have a higher minimum coverage (5X) and takes the allele represented by the majority of reads as the final call. This experiment (Peru.PP.2rnd.>5X.maj, Fig 4) yielded a dramatic shift of the f_4 results towards large negative values, further exacerbating the artefactual shared drift and bias. This is strong evidence that the bias is conferred by the bait design, and results in the persistent overrepresentation of one allele over the other at heterozygous sites. Concordantly, our random diploid genotype test produced even more biased f_4 statistics, such that the estimated levels of shared drift observed between the Spanish PP data and Peruvian PP data that used random diploid genotype calls is of a similar magnitude to the true shared drift between Spanish PP generated and Spanish shotgun data. This is evidence of a

785 remarkably strong systematic bias that has the potential to corrupt all paleogenomic
786 studies combining PP with other data types if not handled carefully. These two results are
787 strongly suggestive of a systematic allelic bias that impacts all heterozygous SNPs and is
788 potentially caused by the molecular design of the PP baits.
789
790 *Laboratory protocol experiments*
791 Our two experiments with single-round PP enrichment yielded f_4 values closer to zero
792 than those produced by the standard two-round enrichment. This improvement is likely
793 because whatever bias occurs during the first round of bait–DNA binding becomes
794 amplified during the second hybridisation step because it is acting on an already biased set
795 of DNA fragments. This interpretation is supported by the sequencing statistics (Fig S5)
796 showing that repeated capture rounds result in an increased endogenous DNA percentage
797 but decreased library complexity (observed as fewer unique reads when sequenced).
798 Additionally, any biased sampling that has occurred during the initial binding will be
799 exponentially amplified during the PCR step, so we would expect a reduction in PCR
800 cycles to improve this. The reduced hybridisation temperature should also decrease the
801 specificity of hybridisation and thus reduce allelic bias, but simultaneously increase off-
802 target binding, thus reducing the percentage of endogenous DNA enriched.
803
804 ***Characterising the bias***

805 Given the evidence that the bias affects all targeted SNPs, we investigated whether this
806 effect was independent from the SNP transition, hoping that we might be able to apply
807 some relatively simple statistical scaling of the genotype calls that would provide co-
808 analysable genotypes. However, our investigations of allelic bias (Fig 5) clearly show that
809 the capture bias produces complex effects on allele calls. We can only confidently observe
810 that the pattern of GC bias in PP data is different to the 1240k and shotgun data. Further,
811 the patterns of missingness evident in the PCA analyses consistently differ between data
812 types, indicating that there may be several layers of biochemical bias at play including
813 that each assay preferentially captures certain SNPs, and/or favours the capture of
814 different alleles at heterozygous sites, with allelic preference being more exacerbated in
815 PP data.
816
817 ***Study limitations***
818 The main limitation of this study is that we did not have samples for which data was
819 generated in all of the three methods addressed. This is due to the opportunistic nature of
820 the study in that we collated this dataset when the PP bias was initially observed, after the
821 data generation process. However, we have made every effort to combat this limitation by
822 maximising the size of the dataset and explicitly stating assumptions where we've made
823 them.
824

825 In our READ analyses we were limited to a dataset of only four individuals for which we
826 had shotgun and PP data, and a further limitation is that this PP data was from one of our
827 single-round enrichment experiments and thus does not fully represent the PP bias under
828 investigation. To further understand the impact on READ, both shotgun and two-round
829 PP enriched data would need to be generated for a larger number of samples, from a more
830 diverse set of relationships to test the impact of bias on truly related individuals.
831
832 The main outstanding hypothesis to investigate is whether this bias has differential effects
833 on different ancestries. This would require a large dataset of PP and shotgun data from the
834 same individuals across more ancestries than in the present study. While an interesting
835 question, we believe this study presents sufficient evidence that the PP assay is biased.
836
837 *Implications*
838 For the past decade in-solution hybridisation enrichment has been a go-to method for
839 obtaining affordable, high-quality, and informative data for paleogenomic studies.
840 However, irrespective of the bait design, the biochemistry of DNA hybridisation means
841 that one bait will always have a higher binding affinity with its targeted allele than any
842 other. Greater differences in these affinities result in increased allelic biases in the
843 generated datasets. This is evidenced by our assessment of alternative enrichment
844 protocols, where the single-round enrichments have less endogenous DNA (Fig S3), but

also appear to be less biased (Fig 4). Therefore, we hypothesise that the enrichment efficiency of the PP reagent is in direct trade-off with the biochemical allelic bias.

It is worth reiterating here that all capture methods used in paleogenomic studies will lead to ascertainment bias, meaning that one must carefully consider both the selection of methods and the use of comparative published data. Previously, the monopoly on data generation by labs with access to the legacy 1240k enrichment protocol somewhat insulated the community from this compatibility issue. However, now that data can be generated using different enrichment kits, researchers face a new set of challenges whenever they integrate data generated with new capture assays with previously published data generated using the legacy 1240k—the most comprehensive dataset to date that underpins many of the findings in paleogenomics. Additionally, researchers utilising the Twist reagent should benchmark the data similarly to the present study to establish its co-analysability with other data types.

Here, we have tested some of the most used analyses in paleogenomic studies, though not an exhaustive list. Principal Components Analysis is a standard way to understand population structure, and outgroup-f_3 and f_4 statistics, along with their more complex derivatives, are integral to reconstructing population relationships and evolutionary history, while READ is a standard for genetic relatedness analysis. We have found that PP-enriched data bias the results of all these analyses when used in combination with other non-PP datasets, and therefore we caution potential PP users that

867 analyses of such multi-assayed datasets may lead to erroneous conclusions if not
868 interpreted carefully.
869
870 Having comparative shotgun data for a subset of samples in a study, or from samples
871 expected to have a very similar ancestry should allow researchers to estimate and control
872 for the PP bias in the specific methods they wish to utilise. Further, while we explicitly
873 test PP data, we advise researchers to exhibit similar caution when using the Daicel Arbor
874 Biosciences Expert Human Affinities "Complete" and "Ancestral Plus" enrichment kits, as
875 the bait chemistry and enrichment protocol is the same as the PP kit, and only the
876 number of target loci differ.
877
878 *Guidelines*
879 - When calculating f-statistics only compare Prime-Plus-generated data with other
880 Prime-Plus-generated data; OR
881 - Only compare one Prime-Plus-generated target population/individual with other
882 non-Prime-Plus-generated populations/individuals (See Table S4 for a full
883 description).
884 - Beware that Prime-Plus-generated data attract each other and that this may
885 artificially confirm/reject your hypothesis depending on the study design.
886 - Explicitly assess the impact of Prime-Plus bias in your data when running analyses
887 that have not yet been investigated.

- Recommend to shotgun sequence a subset of enriched libraries as a control set if possible, ideally select libraries to represent a range of ancestries.
- Always annotate your data as Prime-Plus-generated when publishing, sharing with other researchers, or incorporating into any public database.
- Always annotate the data type of both newly generated and published data used in your study so this can be factored into interpretations.
- For best results compute PCA using EMU and smartPCA and compare results; if clustering appears driven by capture assay, pursue alternative analyses.
- In general, pursue multiple lines of evidence to support your findings.

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Author Contributions

R.D., B.L., L.F-S., M.P.W. and X.R-R. conceptualised the study. R.D., L.F-S., K.K., M.P.W. and X.R-R. performed laboratory work. R.D. and L.F-S. performed computational analyses. R.T., B.L., and L.F-S. funded the study. All authors contributed to manuscript writing and revisions.

1132 **Data Accessibility and Benefit-Sharing Section**

1133 Genomic Data:

1134 The newly generated genomic data used in this study will be made available upon request,

1135 only to replicate the results presented in this study.

1136

1137 Code Availability:

1138 Code used for analyses and plot generation is available at https://github.com/roberta-

1139 davidson/Davidson_etal_2023-Capture-bias-paper.

1140

1141 **Tables and Figures**

1142 **Table 1:** Relevant modified variables for each of the laboratory experiments.

Variable	Standard Protocol	Experiment A	Experiment B
Enrichment rounds	2	1	1
Hybridisation temperature	70°C	70°C	62.5°C
Amplification cycles	20 (1st round) + 10 (2nd round)	12	13

1143

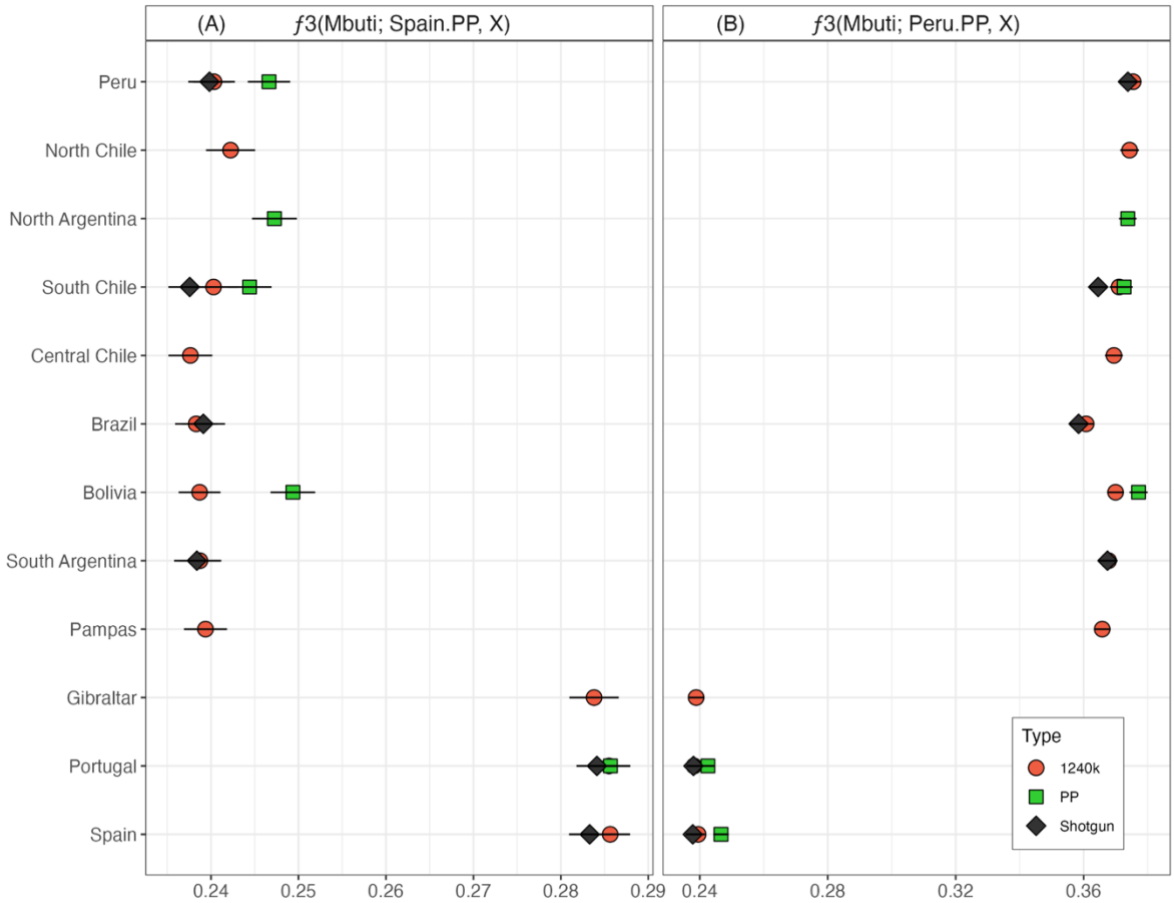


Figure 1: Outgroup f_3 statistics comparing ancient South American and European populations by data type. Panels (A) and (B) show results for Spain.PP.2rnd and Peru.PP.2rnd as the target population, respectively. Note that not all regions have data of every type and different data types from the same region are not the same individuals but are expected to share broadly similar ancestries.

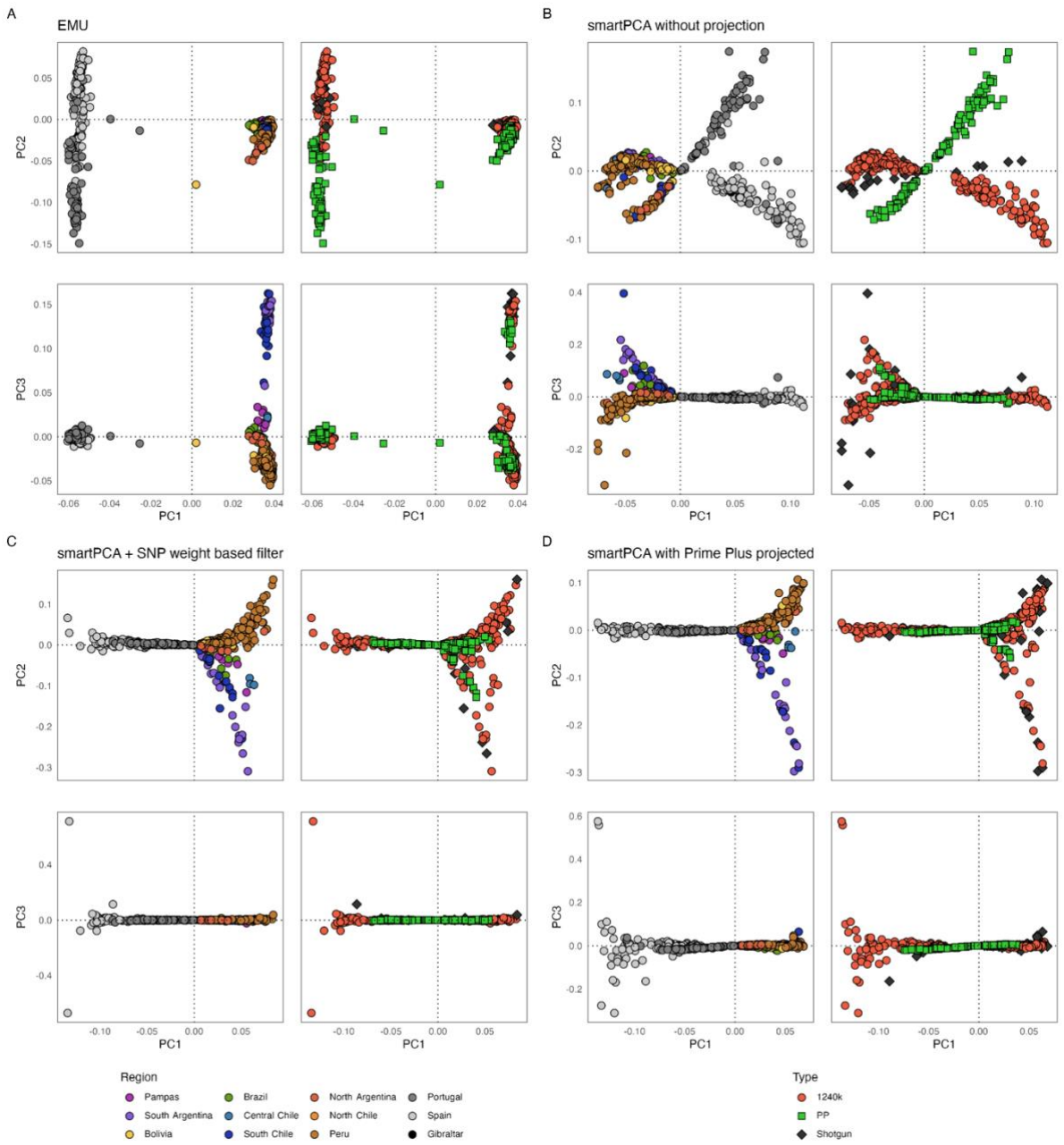


Figure 2: Comparing PCA methods using a collated dataset of 493 ancient genomes from South America and Iberia. Samples are coloured by region (left column of each panel) or data type (right column of each panel). (A) PCA computed with EMU (Meisner et al., 2021). (B) PCA computed with smartPCA, no projection (Patterson et al., 2006; Price et al., 2006). (C) PCA computed on data passed through a PCA-based filter—which removed SNPs that drove PC2 in panel B—using smartPCA without projection (Patterson et al., 2006; Price et al., 2006). (D) PCA computed with smartPCA where Prime Plus data (PP; green squares) were projected onto non-PP data.

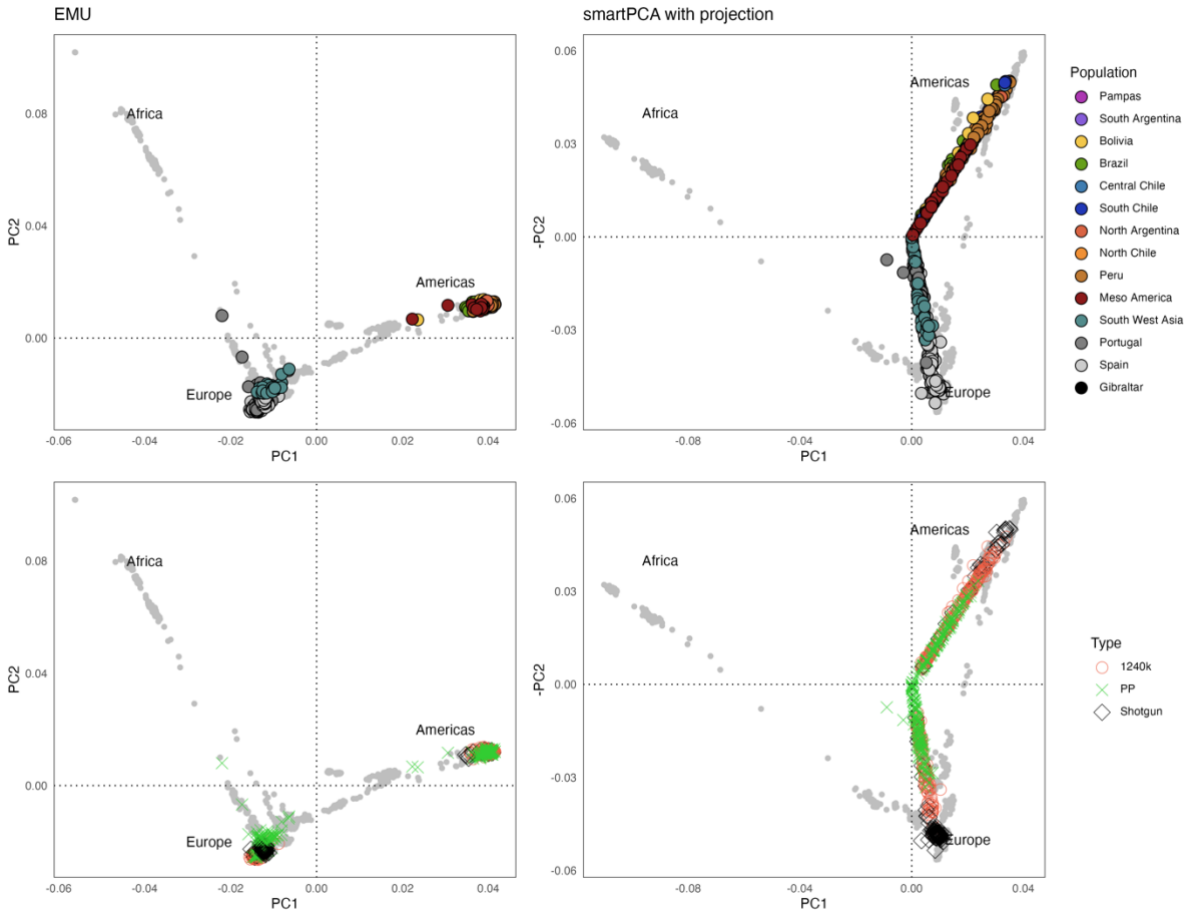
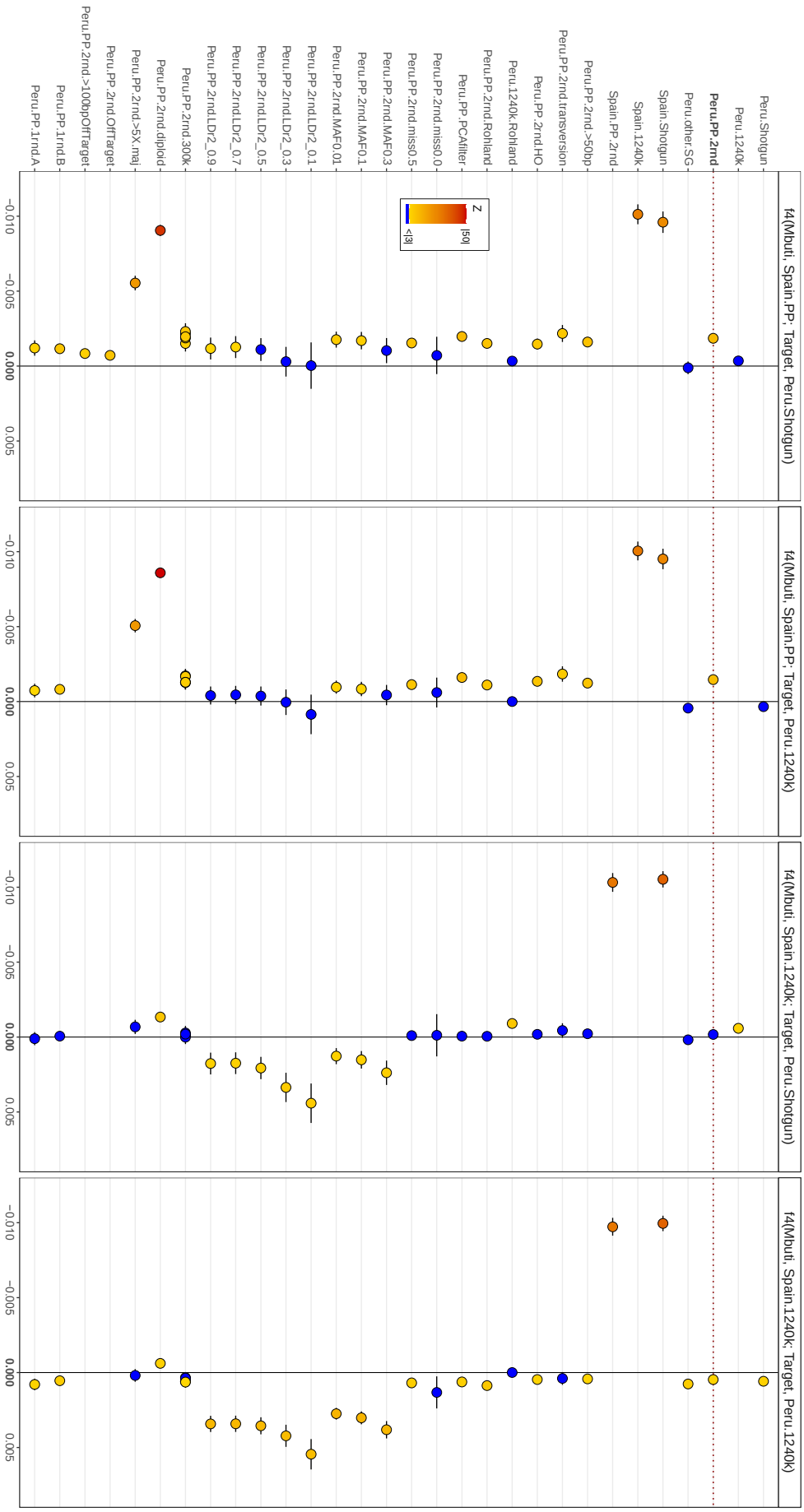


Figure 3: PCAs computed with EMU (left) and smartPCA (right) of ancient genomes from South America, Central America, Southwest Asia and Iberia coloured by region (top row) or data type (bottom row). Ancient samples are projected by smartPCA onto the modern diversity of the HGDP shown in grey (Bergström et al., 2020).


f4(Mbuti, Spain.PP; Target, Peru.Shotgun)
f4(Mbuti, Spain.PP; Target, Peru.1240k)
f4(Mbuti, Spain.1240k; Target, Peru.Shotgun)
f4(Mbuti, Spain.1240k; Target, Peru.1240k)
Peru.Shotgun
Peru.1240k
Peru.PP.2rnd
Peru.other.SG
Spain.Shotgun
Spain.1240k
Spain.PP.2rnd
Peru.PP.2rnd.>50bp
Peru.PP.2rnd.transversion
Peru.PP.2rnd.HO
Peru.1240k.Rohland
Peru.PP.2rnd.Rohland
Peru.PP.PCAfilter
Peru.PP.2rnd.miss0.0
Peru.PP.2rnd.miss0.5
Peru.PP.2rnd.MAF0.3
Peru.PP.2rnd.MAF0.1
Peru.PP.2rnd.MAF0.01
Peru.PP.2rnd.LDr2_0.1
Peru.PP.2rnd.LDr2_0.3
Peru.PP.2rnd.LDr2_0.5
Peru.PP.2rnd.LDr2_0.7
Peru.PP.2rnd.LDr2_0.9
Peru.PP.2rnd.300k
Peru.PP.2rnd.diploid
Peru.PP.2rnd.>5X.maj
Peru.PP.2rnd.OffTarget
Peru.PP.2rnd.>100bpOffTarget
Peru.PP.1rnd.B
Peru.PP.1rnd.A
Z
|5|
<|3|
−0.010
−0.005
0.000
0.005

1165

Figure 4: f_4 statistics showing the effectiveness of 29 bias-reduction strategies. Test configurations are, from left to right: f_4(Mbuti, Spain.PP.2rnd; Target, Peru.Shotgun), f_4(Mbuti, Spain.PP.2rnd; Target, Peru.1240k), f_4(Mbuti, Spain.1240k; Target, Peru.Shotgun), f_4(Mbuti, Spain.1240k; Target, Peru.1240k). "Target" groups are shown along the y-axis, being either one of the 29 strategies used to resolve the Prime Plus bias, or shotgun or 1240k data from Peru or Spain. Numbers above the points indicate the number of SNPs used in each f_4 statistic and the associated Z-score. Non-significant Z-scores ($|Z| < 3$) are shown in blue, and significant Z-scores ($|Z| > 3$) are indicated with a yellow-red gradient. Error bars represent ± 2 se. The vertical dashed line represents the observed f_4 statistic in each configuration when Peru.PP.2rnd was the Target group. Note that bias-reduction strategies are only applied to the target population and the f_4 statistic is calculated from SNPs overlapping between the target and the other populations that remain unchanged between tests. Note that in the f_4 statistics where the target population is off-target or out-of-range genotypes (Peru.PP.2rnd.OffTarget, Peru.PP.2rnd.>100bpOffTarget), all four populations were genotyped in the respective alternative way to ensure overlapping SNPs, and therefore these are not exactly comparable.

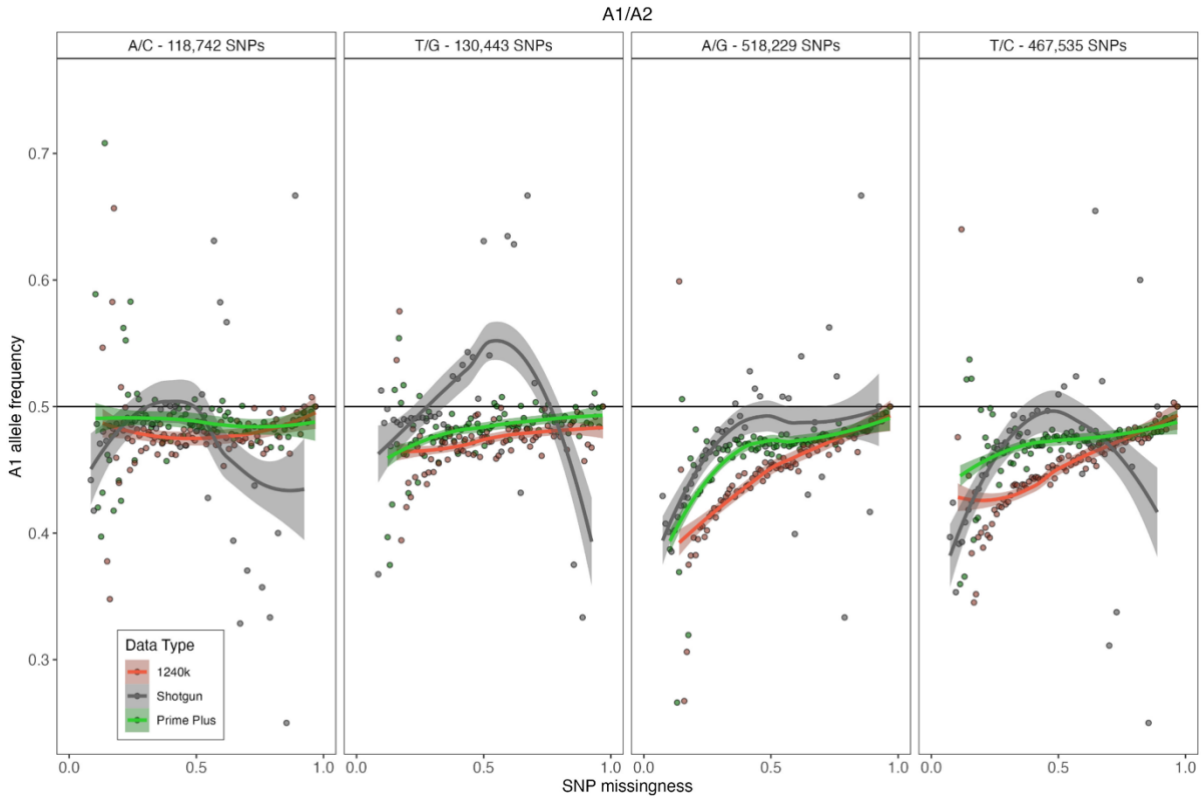


Figure 5: Mean frequency of A1 allele calls in different data types as a function of SNP missingness. Results for the four different A1 allele pairs are provided in separate panels, with inset data points showing the mean allele frequencies calculated across all individuals in successive SNP missingness bins. SNPs that are fixed within each dataset were removed

1189 to avoid skewing the mean A1 calls. The header of each panel shows the number of target
1190 SNPs of the corresponding A1/A2 pair observed in the Prime Plus assay.

Supplementary Information

Allelic bias when performing in-solution enrichment of ancient human DNA

Roberta Davidson, Matthew P. Williams, Xavier Roca-Rada, Kalina Kassadjikova, Raymond Tobler, Lars Fehren-Schmitz, Bastien Llamas

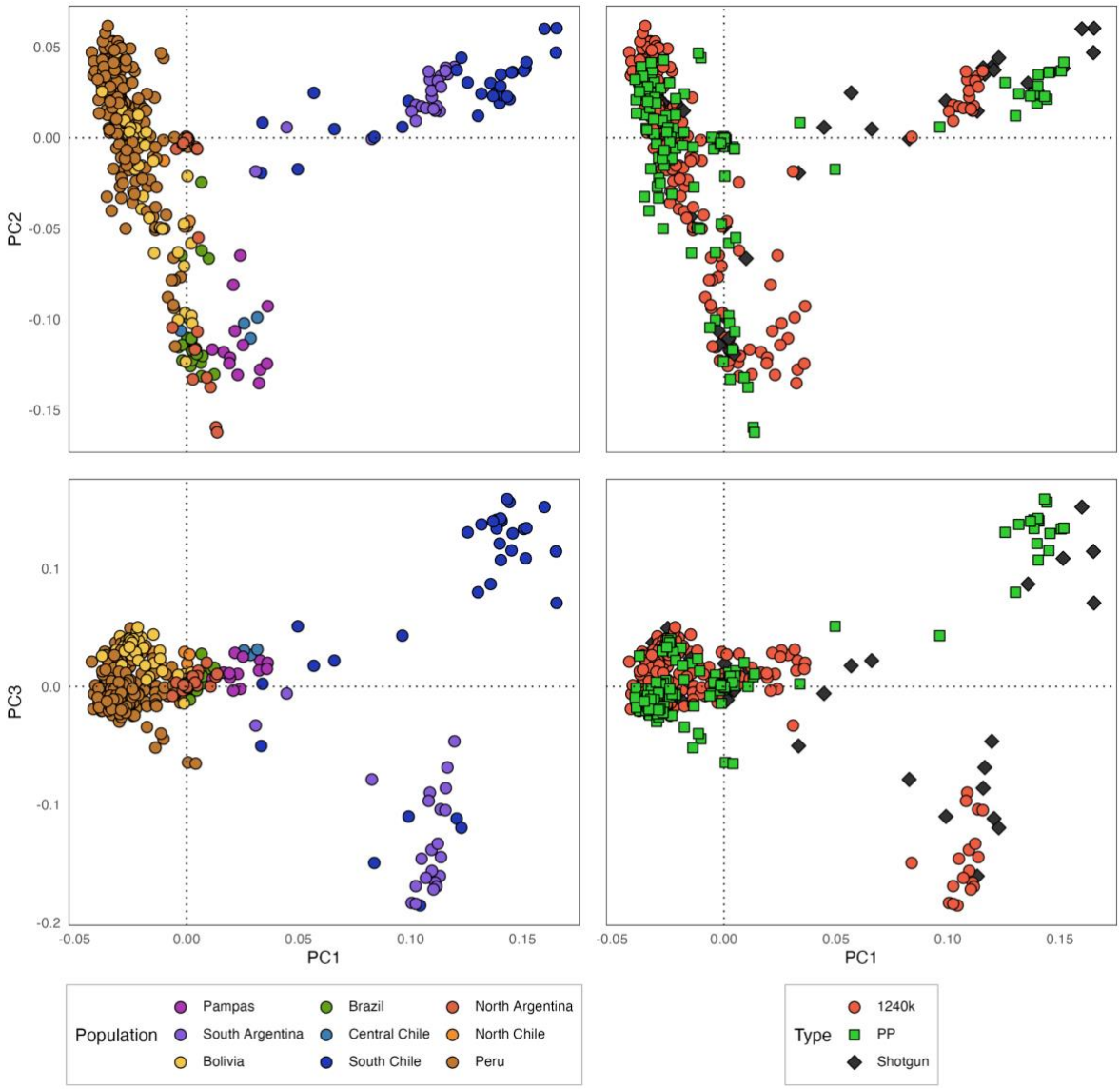


Figure S1: PCA of ancient genomes from South America coloured by region (left) and data type (right). Shapes are consistent between rows. Computed with EMU (Meisner et al., 2021).

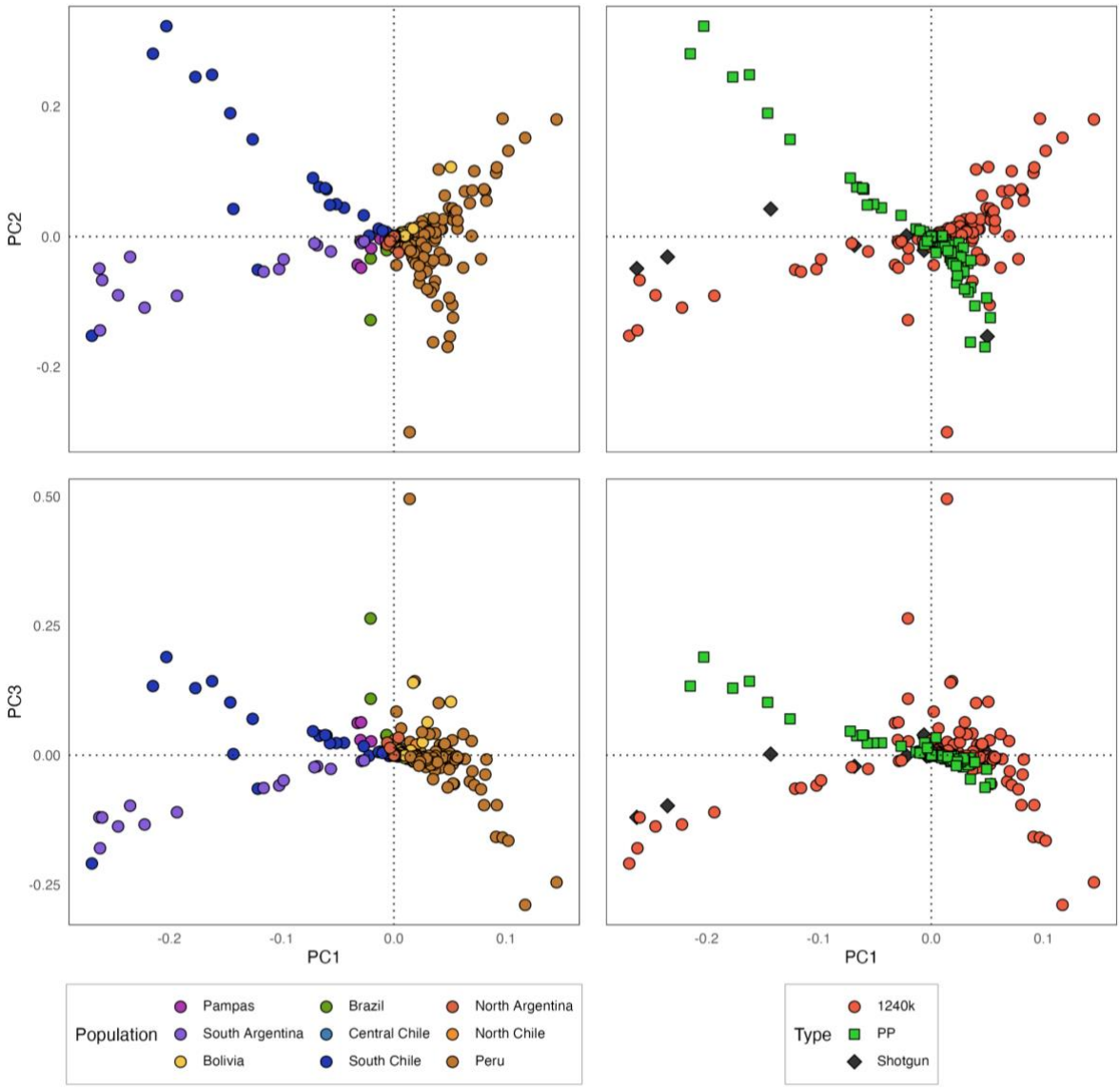


Figure S2: PCA of ancient genomes from South America coloured by region (left) and data type (right). Shapes are consistent between rows. Computed with smartPCA without projection (Patterson et al., 2006; Price et al., 2006).

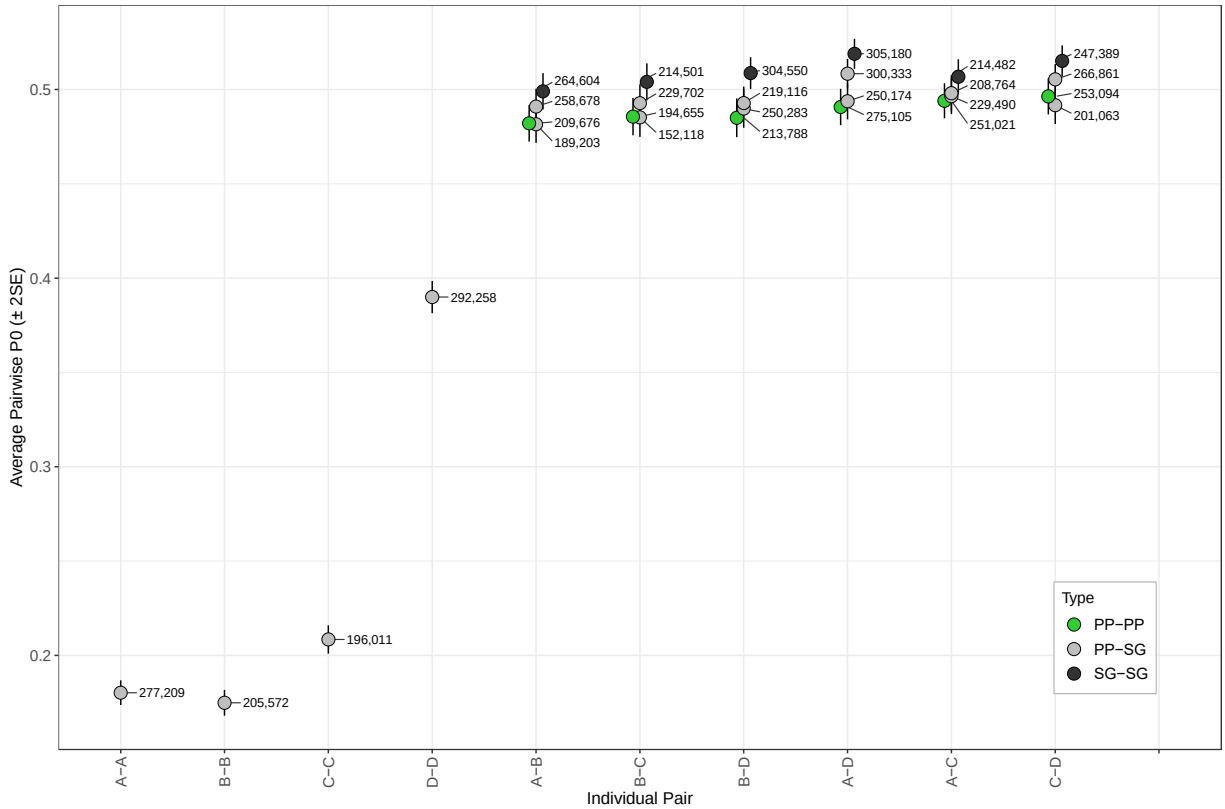


Figure S3: READ results for pairwise combinations of four individuals from Peru for which we had generated shotgun and single round Prime Plus enriched data as a part of Experiment B. Each point is labelled with the number of SNPs used in the pairwise comparison.

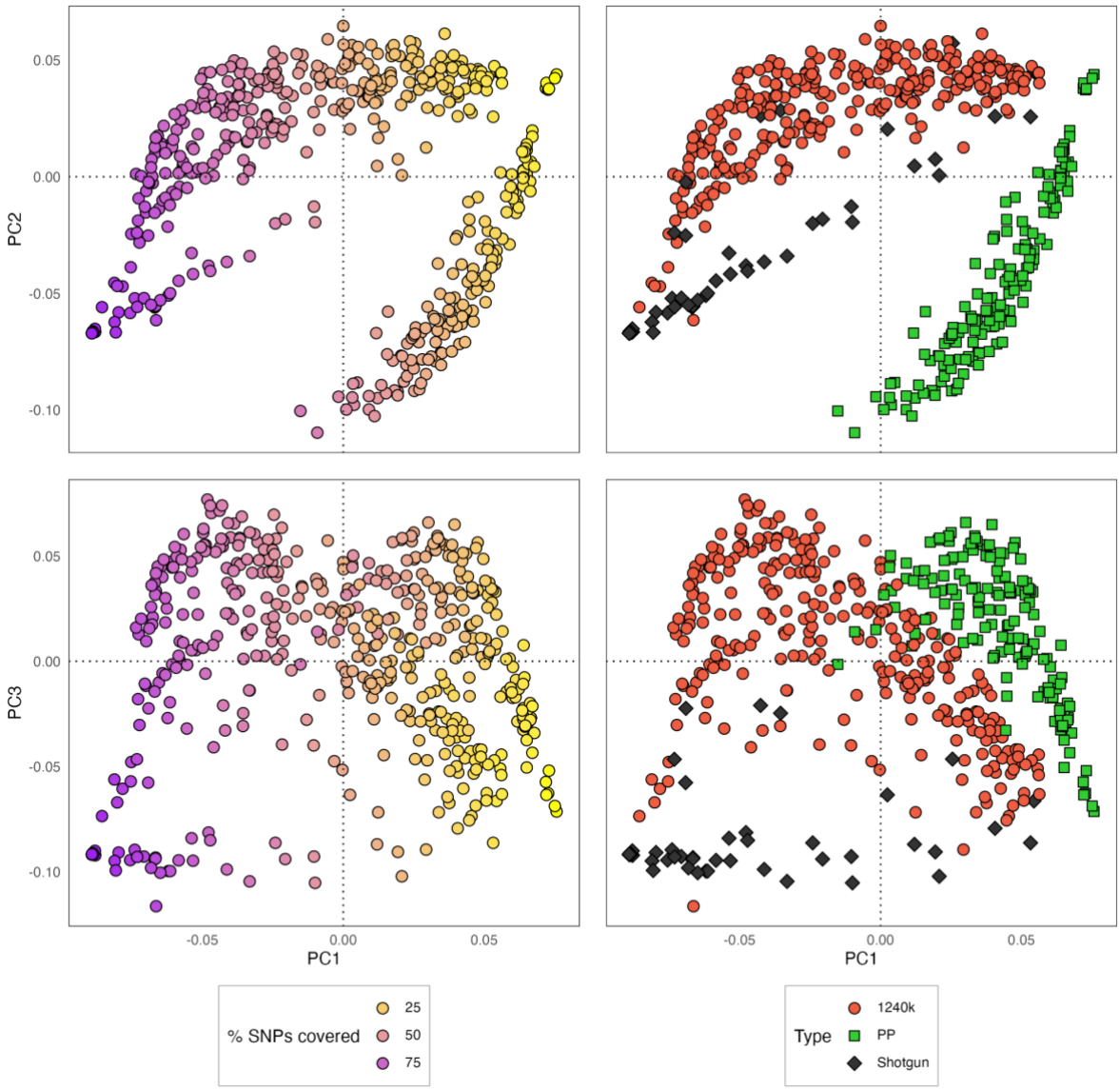


Fig S4: PCA of SNP missingness between data types (bottom row) computed with smartPCA. The top row is coloured by missingness frequency where yellow is high missingness and blue is low missingness.

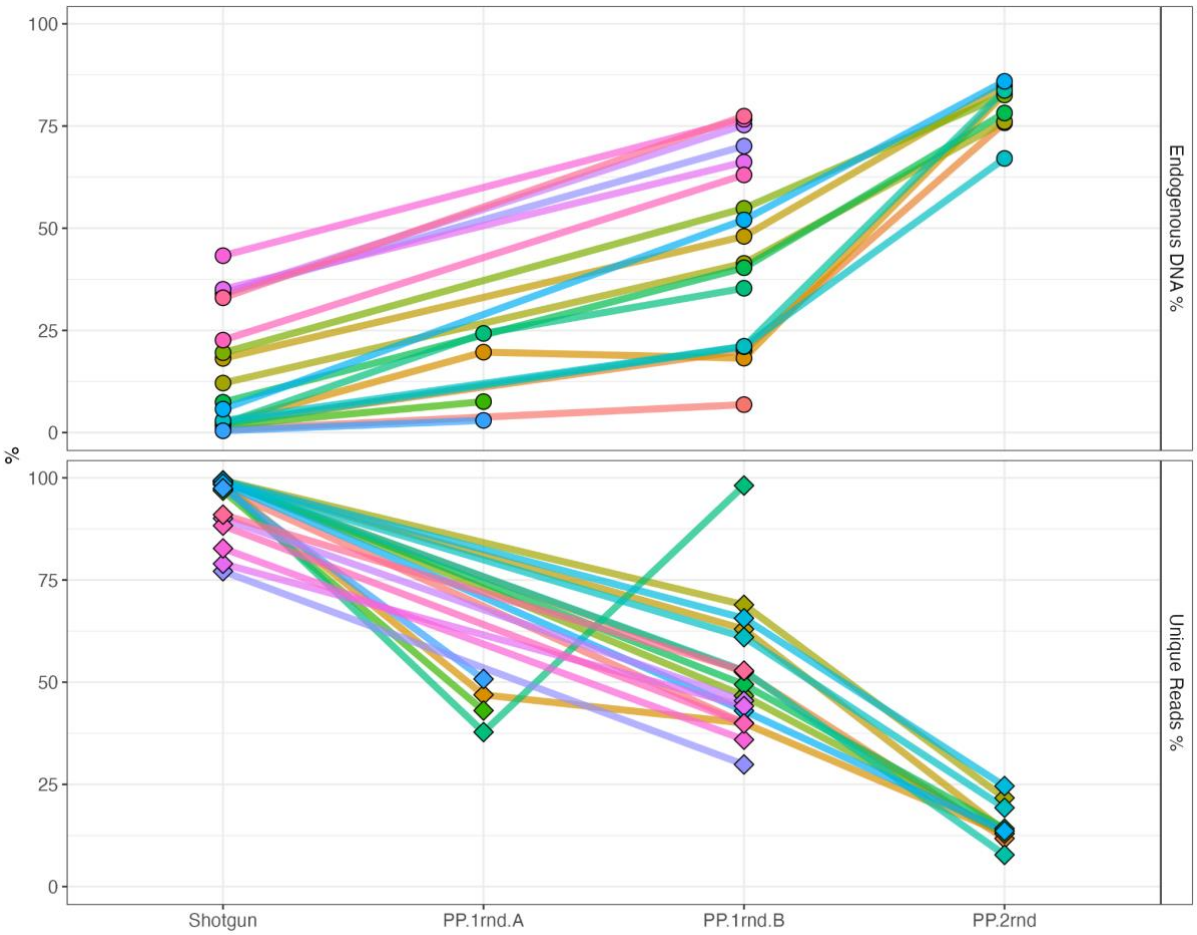


Figure S5: Key summary statistics from sequencing libraries at different levels of enrichment (x-axis), contrasting increasing endogenous DNA retrieved (top) with decreasing complexity, taken as % unique reads (bottom). Values are standardised by the total reads.

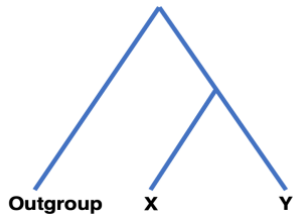
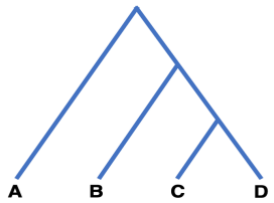
Tables S1-S3 and S6 are in the Supplementary Excel spreadsheet.

Table S1: Number of ancient samples per region for each data type represented in this study.

Table S2: Processing details of all Prime Plus enriched samples in this study.

Table S3: Metadata for all published samples used as comparative data in this study

Table S4: List of possible ways to structure outgroup-f_3 and f_4 statistics as well as qpAdm and qpWave configurations using PP data and their respective reliability.

***f*₃ statistic structure**	**Reliability and Expectation**
	*Where Outgroup is kept constant between comparisons and X and/or Y rotate between populations or individuals*
f_3 (Outgroup.PP; X.PP, Y.PP)	Reliable, bias affects all equally
f_3 (Outgroup.notPP; X.PP, Y.PP)	Reliable, under the assumption that bias does not significantly differentially affect different ancestries.
f_3 (Outgroup.notPP; X.sometimesPP, Y.sometimesPP)	Unreliable, X.PP will always have more shared drift with Y.PP than X.notPP.
f_3 (Outgroup.notPP; X.notPP, Y.PP)	Reliable, the bias in Target.PP is not shared with X or Outgroup so the test should be agnostic to it.
f_3 (Outgroup.notPP; X.notPP, Y.notPP)	Reliable when using shotgun or legacy 1240k, standard practice.
***f*₄ statistic structure**	**Reliability and Expectation**
	*Where A is usually an outgroup population and B, C and D may rotate between populations or individuals*
f_4 (A.PP, B.PP; C.PP, D.PP)	Reliable, bias affects all equally
f_4 (A.notPP, B.PP; C.PP, D.PP)	Reliable, under the assumption that bias does not significantly differentially affect different ancestries.
f_4 (A.notPP, B.PP; C.notPP, D.PP)	Unreliable, B.PP and D.PP will be artificially attracted to each other
f_4 (A.notPP, B.notPP; C.PP, D.PP)	Reliable, as C and D share the PP bias, the test will reveal whether either received gene flow from B.
f_4 (A.notPP, B.PP; C.PP, D.notPP)	Unreliable, C.PP will be more attracted to the other PP population than it truly is.

f_4(A.notPP, B.notPP; C.sometimesPP, D.PP) f_4(A.notPP, B.notPP; C.PP, D.sometimesPP)	Unreliable
f_4(A.PP, B.notPP; C.notPP, D.notPP) f_4(A.notPP, B.PP; C.notPP, D.notPP) f_4(A.notPP, B.notPP; C.PP, D.notPP) f_4(A.notPP, B.notPP; C.notPP, D.PP)	Reliable, the bias in the one PP population is not shared with the others so the test should be agnostic to it.
f_4(A.notPP, B.notPP; C.notPP, D.notPP)	Reliable when using shotgun or legacy 1240k, standard practice
qpAdm configuration	**Reliability and Expectation**
Where T = target, S = source, R = reference	
(T) = T.PP, (S) = S.notPP, (R) = R.notPP	Reliable, under the assumption that the PP bias does not significantly differentially affect different ancestries or either of the shotgun and 1240k technologies.
(T) = T.PP, (S) = S.sometimesPP, (R) = R.notPP	Unreliable, bias in the matrix of f_4 statistics underlying qpAdm could lead to artificial relationships and weight estimations from the source to the target population.
(T) = T.PP, (S) = S.sometimesPP, (R) = R.sometimesPP	Unreliable, bias in the matrix of f_4 statistics could lead to incorrect estimation of the matrix rank in addition to the problems outlined above.
(T) = T.PP, (S) = S.notPP, (R) = R.sometimesPP	Unreliable, bias in the rank of the matrix of f_4 statistics can be driven by the artificial affinity between the T.PP target and R.PP right-group leading to a false inference in an increase in the matrix rank.
qpWave configuration	**Reliability and Expectation**
Where L = left, R = right	
(L) = L.PP, (R) = R.PP (L) = L.PP, (R) = R.notPP (L) = L.notPP, (R) = R.PP	Reliable.
(L) = L.PP, (R) = R.sometimesPP (L) = L.sometimesPP, (R) = R.PP (L) = L .sometimesPP, (R) = R	Unreliable, bias in the matrix of f_4 statistics between L.PP and R.PP underlying qpWave could lead to a false estimation of the matrix rank.

.sometimesPP	

Table S5: List of all strategies tested to reduce Prime Plus assay bias and their predicted impact on bias. Filters tried with different thresholds have variable values listed inside braces {}.

Strategy	**Hypothesised impact or reason for testing.**
Remove transition SNPs	Remove possible impact of ancient DNA damage.
Remove reads <50 bp	Reduce impact of reference bias.
Allele frequency-based filters	
SNP missingness {>0%, >50%}	Reduce impact of bias caused by preferential capture of certain SNPs by each enrichment, causing differential patterns of data missingness.
Minor allele frequency (MAF) {> 1%, >10%, >30%}	Explore the effect of MAF filters on bias. Informative about the relationship of bias to segregating sites.
Linkage disequilibrium filters Window size = 3 variants, slide = 1 variant, r^2 = {0.1,0.3,0.5,0.7,0.9}	Remove artefactual 'linkage' between neighbouring variants that could potentially be captured by one bait. Exploratory tested with a range of r^2 values due to unknown expected correlation.
SNP-set based filters	
Rohland et al. filter	Reported in Rohland et al. 2022 to allow co-analysis of Legacy 1240k and shotgun data.
Filter to Human Origins SNPs	Explorative, to understand the bias impact on these most commonly used SNPs
PCA-derived filter	Remove the bias conferred by SNPs driving the "assay bias".
Alternative genotyping	
Random diploid	Explore the capture of alleles at heterozygous sites.
Pseudohaploid by majority (>5X sites)	
Off-target sites	Remove bias that could be due to the biochemical binding of bait to target DNA, focussing on off-target by-catch.
>100 bp out of range sites	Same as above, plus removing biased by-catch where one bait may cover two target sites.
Laboratory protocol adjustments	
Protocol A Single capture round, less amplification cycles	Overall reduction of PCR cycles reduces amplification of any preferential binding bias.
Protocol B Single capture round, less amplification cycles, lower	Same as above, and lower hybridisation temperature decreases specificity of bait binding, thus reducing bias in the allele captured.

hybridisation temperature	

Table S6: Count of number of SNPs used in each f_4 test in Figure 4 and corresponding Z scores.