

The presence and impact of reference bias on population genomic studies of prehistoric human populations

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Running title: Reference bias in ancient DNA data

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Abstract

High quality reference genomes are an important resource in genomic research projects. A consequence is that DNA fragments carrying the reference allele will be more likely to map successfully, or receive higher quality scores. This reference bias can have effects on downstream population genomic analysis when heterozygous sites are falsely considered homozygous for the reference allele.

In palaeogenomic studies of human populations, mapping against the human reference genome is used to identify endogenous human sequences. Ancient DNA studies usually operate with low sequencing coverages and fragmentation of DNA molecules causes a large proportion of the sequenced fragments to be shorter than 50 bp – reducing the amount of accepted mismatches, and increasing the probability of multiple matching sites in the genome. These ancient DNA specific properties are potentially exacerbating the impact of reference bias on downstream analyses, especially since most studies of ancient human populations use pseudo-haploid data, i.e. they randomly sample only one sequencing read per site.

We show that reference bias is pervasive in published ancient DNA sequence data of prehistoric humans with some differences between individual genomic regions. We illustrate that the strength of reference bias is negatively correlated with fragment length. Reference bias can cause differences in the results of downstream analyses such as population affinities, heterozygosity estimates and estimates of archaic ancestry. These spurious results highlight how important it is to be aware of these technical artifacts and that we need strategies to mitigate the effect. Therefore, we suggest some post-mapping filtering strategies to resolve reference bias which help to reduce its impact substantially.

Introduction

1 The possibility to sequence whole genomes in a cost-efficient way has revolutionized the way how
2 we do genetic and population genetic research. Annotated, high-quality reference genomes are a
3 cornerstone for resequencing surveys which aim to study the genetic variation and demographic
4 history of an entire species. Resequencing studies usually align the sequences of all studied in-
5 dividuals to a linear haploid reference sequence originating from a single individual or a mosaic
6 of several individuals. In each site, this haploid sequence will only represent a single allele out
7 of the entire genetic variation of the species. An inherent consequence is some degree of bias to-
8 wards the alleles present in that reference sequence (“reference bias”). Sequencing reads carrying

9 an alternative allele will naturally have mismatches in the alignment to the reference genome and
10 consequently have lower mapping scores than reads carrying the same allele as the reference. This
11 effect increases with genetic distance from the reference genome, which is of particular interest
12 when using a reference genome from a related species for mapping (Shapiro and Hofreiter, 2014;
13 Gopalakrishnan et al., 2017; Heintzman et al., 2017). Generally, reference bias can influence vari-
14 ant calling by missing alternative alleles or by wrongly calling heterozygous sites as homozygous
15 reference (Bobo et al., 2016; Ros-Freixedes et al., 2018) which is known to influence estimates of
16 heterozygosity and allele frequencies (Chen et al., 2012; Bryc et al., 2013; Brandt et al., 2015).

17 The field of palaeogenomics and the population genomic analysis of DNA obtained from hominin
18 remains has led to a number of important insights and groundbreaking results in recent years,
19 including admixture between different hominin groups, migrations of prehistoric humans and the
20 evolution of different phenotypes (Günther and Jakobsson, 2016; Slatkin and Racimo, 2016; Nielsen
21 et al., 2017; Dannemann and Racimo, 2018; Lazaridis, 2018; Skoglund and Mathieson, 2018). DNA
22 preservation poses a major challenge for these studies, as fragmentation causes most authentic
23 sequences to be shorter than 100 bp, and deamination damage increases the number of mismatches
24 and can even mimic genetic variation at transition sites (Hofreiter et al., 2001; Brotherton et al.,
25 2007; Briggs et al., 2007).

26 In addition to fragmentation and other post-mortem damages, low coverage data is a major
27 limiting factor for ancient DNA studies. Coverages below 1x rarely permit calling diploid genotypes
28 so a very common approach is to use “pseudo-haploid” data: at each known single nucleotide
29 polymorphisms (SNP) site one sequencing read is picked at random (or following a majority rule)
30 in order to represent a haploid genotype of that individual. This approach would not introduce bias
31 if the reads were a random representation of the chromosomes carried by the individual. Reference
32 bias, however, would introduce some skew towards the reference allele at heterozygous sites. These
33 characteristics of ancient DNA and practices used in palaeogenomic studies make them particularly
34 vulnerable to reference bias (Prüfer et al., 2010; Schubert et al., 2012). It has been shown that
35 pseudo-haploid data can be more biased than imputed genotypes (Martiniano et al., 2017), and
36 that reference bias and fragment length artifacts can interfere with phylogenetic classifications
37 (Heintzman et al., 2017). Reference bias can influence downstream analyses if these are based on
38 estimating allele frequencies in a population, or studying pairwise allele sharing between individuals
39 and groups.

40 This study investigates the presence and impact of reference bias in studies of prehistoric
41 human populations using genomic ancient DNA. We first illustrate its abundance in published
42 data from ancient human and archaic hominins, and illustrate how it is influenced by standard
43 data processing. We then show how reference bias can influence some basic population genetic

44 analyses such as population affinities and heterozygosity. Finally, we discuss some possible data
45 filtering strategies in order to mitigate reference bias in ancient DNA studies.

46 Results

Table 1: Information on the published medium to high coverage palaeogenomic and archaeogenomic data used in this study.

Sample ID	(Partial) UDG treatment [§]	SNP capture	Average sequencing depth [†]	Reference
Stuttgart	X		15.8x	Lazaridis et al. (2014)
Loschbour	X		17.7x	Lazaridis et al. (2014)
Ust-Ishim	X		29.9x	Fu et al. (2014)
sf12	X		64.7x	Günther et al. (2018)
baa001	X		13.2x	Schlebusch et al. (2017)
Kotias			12.5x	Jones et al. (2015)
Bichon			15.4x	Jones et al. (2015)
ne1			18.5x	Gamba et al. (2014)
br2			15.4x	Gamba et al. (2014)
atp016			14.1x	Valdiosera et al. (2018)
Rathlin1			10.9x	Cassidy et al. (2015)
Ballynahatty			10.7x	Cassidy et al. (2015)
I0054	X	X	2.4x	Mathieson et al. (2015)
I0103	X	X	2.4x	Mathieson et al. (2015)
I0118	X	X	1.7x	Mathieson et al. (2015)
I0172	X	X	3.4x	Mathieson et al. (2015)
I0408	X	X	1.7x	Mathieson et al. (2015)
I0412	X	X	1.9x	Mathieson et al. (2015)
I0585	X	X	2.4x	Mathieson et al. (2015)
AltaiNeandertal	X		45.2x	Prüfer et al. (2014)
VindijaNeandertal	X		25.9x	Prüfer et al. (2017)
Denisovan	X		26.7x	Meyer et al. (2012)

[§] Enzymatic repair of deamination damages

[†] at analyzed SGDP SNPs, using a minimum mapping quality of 30

47 Mapping quality filtering

48 We first investigate whether reference bias is present in published ancient DNA data. We restrict
49 our analysis to known SNPs, as most population genomic analyses are using SNPs and the allele
50 frequencies at those positions. In particular, we are only using transversion polymorphisms (to
51 avoid the effect of post-mortem deaminations) and sites identified to be polymorphic in a world-wide
52 set of modern human populations (Mallick et al., 2016). We investigate supposedly heterozygous
53 sites (defined as sites covered by at least 10 reads with at least 25% representing the minor allele)
54 in a set of published medium to high coverage human and hominin genomes (Table 1). We note
55 that our approach does not include any rescaling of base qualities, as such approaches usually take
56 the reference allele into account which may amplify reference bias.

57 At a heterozygous site, a DNA extract of an individual should contain the same number of ref-
58 erence and alternative fragments. We observe that after mapping to the human reference genome
59 the average proportion of alternative alleles is lower than the expected 50 percent for all of the
60 anatomically modern humans investigated (Figure 1), regardless of whether they represent SNP
61 capture data, damage repaired libraries or standard shotgun sequencing (Table 1). As sequence
62 fragments carrying the alternative allele will show an elevated number of mismatches to the refer-
63 ence genome, mapping quality seems a natural filter to avoid reference bias. Consistent with this
64 expectation, we see a slightly stronger reference bias for stricter mapping quality filters. Lowering
65 the mapping quality cutoff can have other detrimental effects, however, for example an enrichment
66 of microbial contamination (Renaud et al., 2017) or sequences not uniquely mapping to a particular
67 region of the genome. This is somewhat illustrated by the archaic genomes, two Neandertals and
68 a Denisovan, which show - on average - a bias towards the alternative allele when no mapping
69 quality filter is employed (Figure 1). This suggests that these more distant taxa carry variation in
70 the genome which is not captured by the reference genome based on anatomically modern humans,
71 in turn causing fragments originating in other parts of the genome to map at the investigated sites.
72 As the qualities of the base calls have not been rescaled after mapping to the reference genome, we
73 do not see an effect of different minimum base quality thresholds on reference bias (Supplementary
74 Figure 1).

75 Investigating pairwise correlations between the proportion of alternative alleles at sites con-
76 sidered heterozygous in both individuals shows significantly positive correlations in most cases
77 (Supplementary Table 1). This indicates that the strength of reference (and alternative) bias may
78 differ regionally across the genome, so there could be an effect of sequence context and uniqueness
79 of the specific sequences across the genome. The highest correlations are observed between samples
80 from the same study or produced by the same institute suggesting that similar wet lab techniques
81 also influence this effect.

82 **Distribution of bias**

83 To investigate the distribution of reference bias instead of just averages as above, we modified
84 original reads to carry opposite alleles at each SNP site and remapped them. We created a virtual
85 read set for the Scandinavian Mesolithic hunter-gatherer sf12 (Günther et al., 2018) containing
86 reads for all SNPs identified with mapping quality and base quality of at least 30 in the original
87 mapping. No filter was placed on coverage, a SNP was included even if it was only covered by a
88 single read. This joint read set of original and modified reads thus had perfectly balanced allele
89 ratios for all SNPs. The full set was remapped, and SNPs were grouped based on the observed
90 alternative allele fraction among all reads that again mapped to their respective SNPs with mapping

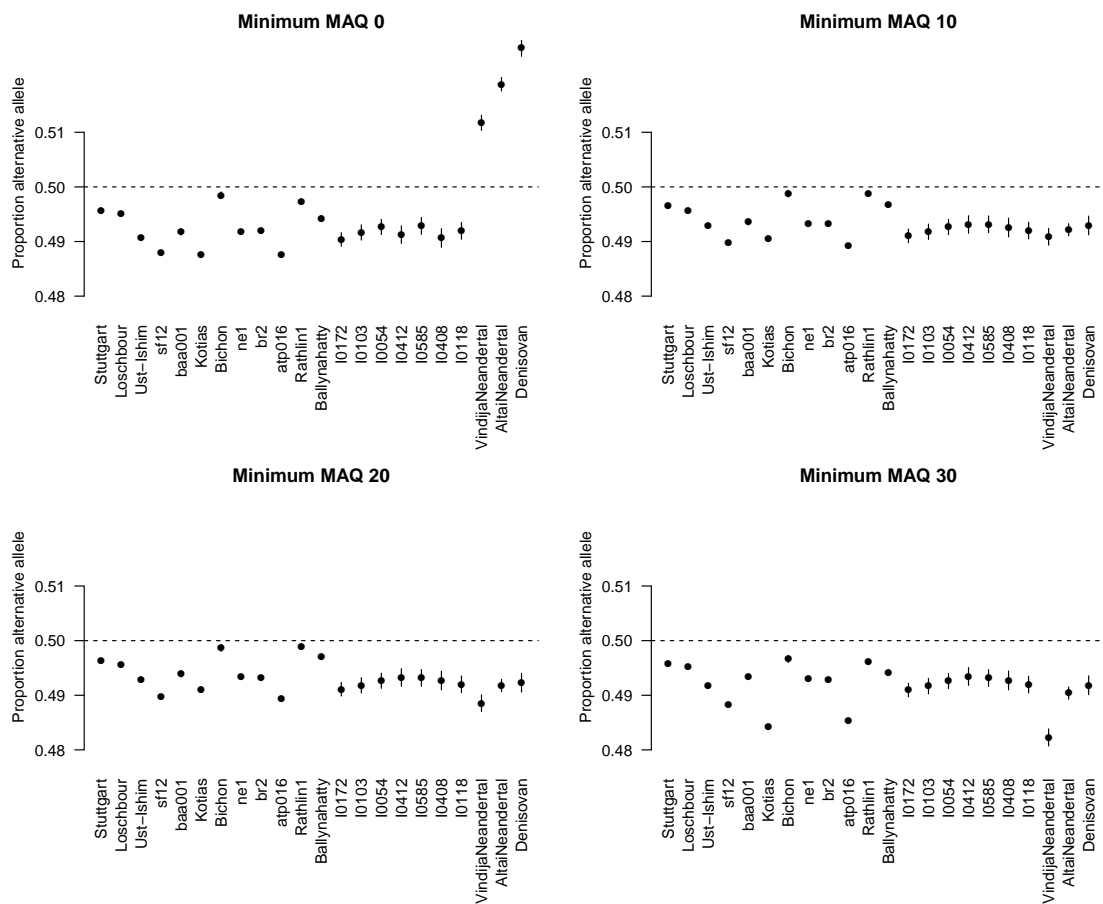


Figure 1: Reference bias in published genome-wide ancient DNA datasets for different minimum mapping quality thresholds. The plots show the average proportion of reads at heterozygous transversion sites (see Methods) representing the alternative allele. Error bars indicate two standard errors of the mean.

91 quality of at least 30.

92 In total, 1,157,266 SNPs were analyzed. Out of these, 1,088,802 (94.08%) showed perfect allelic
 93 balance, with a proportion of alternative alleles of 0.5. About 100 SNPs were also affected by reads
 94 that mapped back with sufficient quality, but to a different genomic location. In these results, we
 95 only present proportions based on reads that map back to their original location from the first
 96 mapping round. The proportion of alternative alleles is summarized in Table 2. Notably, there
 97 is a subset of SNPs showing alternative, as opposed to reference, bias, and also a subset of SNPs
 98 where the bias is total, i.e. only one of the two alleles is ever mapped back successfully (within this
 99 dataset). The distribution across the genome of sites deviating from the balanced case is similar
 100 to the overall density of the SNPs used. Generally, all chromosomes and chromosomal regions are
 101 affected.

Table 2: Proportion of alternative alleles when mapping back original reads and virtual opposite allele reads for the sf12 individual.

Proportion of alternative alleles	# SNPs	Percentage
0	11497	0.99%
(0, 0.4)	12962	1.12%
[0.4, 0.5)	37262	3.22%
0.5	1088802	94.08%
(0.5, 0.6]	4172	0.36%
(0.6, 1)	1413	0.12%
1	1158	0.10%

102 The influence of fragment length

103 Most mapping strategies set the number of allowed mismatches relative to the length of the se-
104 quenced fragment. Therefore, shorter fragments might show a stronger reference bias than long
105 fragments. To investigate this, we used the 57x genome generated for the Scandinavian Mesolithic
106 hunter-gatherer sf12 (Günther et al., 2018) and partitioned the data into fragment length bins.
107 The large amount of data allows us to still have a sufficient number of SNPs covered at 10x or
108 more for each of the length bins.

109 Somewhat expectedly, shorter fragments display a stronger reference bias than longer sequences
110 (Figure 2A). Generally, fragment length might be a main driver of reference bias across all samples
111 as the mode of each individual’s fragment size distribution is highly correlated with the average
112 proportion of alternative alleles at heterozygous sites (Pearson’s $r = 0.67$, $p = 0.0006$; Figure 2B).
113 This also has an effect on the proportion of sites considered heterozygous among all sites analyzed
114 which can be seen as a relative measure for the individual’s heterozygosity (Figure 2C). In fact,
115 different fragment length bins of the same individual produce heterozygosity estimates that do
116 not overlap in their 95% confidence interval (Figure 2C). This represents a general limitation for
117 estimating heterozygosity from ancient DNA data which may to some degree explain the generally
118 low diversity estimates for many prehistoric groups (e.g. Skoglund et al., 2014; Kousathanas et al.,
119 2017; Scheib et al., 2018). The potential of obtaining significantly different estimates for the same
120 population genetic statistic may also have enormous effects on other downstream analyses such as
121 population affinities and population structure.

122 Impact on measures of population affinity

123 In order to investigate the influence of reference bias on population affinities, we calculated dif-
124 ferent combinations of D statistics of the form $D(\textit{Chimp}, X; Y, Z)$, where X is a modern human
125 population, and Y and Z are two different treatments of the same individual sf12. Therefore, the
126 expectation for D is 0, but differences in reference bias between Y and Z could lead to spurious
127 allele sharing between population X and a deviation from 0. Negative values of D indicate more

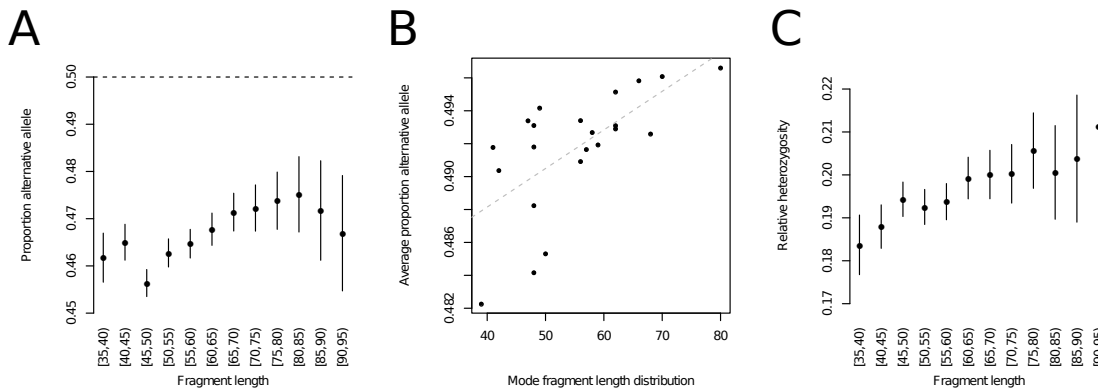


Figure 2: Connection between fragment length and reference bias. (A) Proportion of alternative allele for different fragment length bins in the high coverage individual sf12. (B) Correlation between average proportion of alternative alleles and the mode of the fragment size distribution across all investigated individuals. (C) Proportion of heterozygous sites among all sites with sufficient coverage for different fragment length bins in the high coverage individual sf12. All error bars indicate two standard errors.

128 allele sharing of X with Y while positive values indicate an excess of shared alleles between X
 129 and Z . The populations X were grouped by continental origin and we calculated the statistics
 130 separately for whole genome shotgun data (SGDP, Mallick et al., 2016) and genotyped populations
 131 (HO, Lazaridis et al., 2014).

132 We use four different versions of genotypes for sf12. First, we compare pseudo-haploid calls
 133 (random allele per site with minimum mapping and base quality of 30) to diploid genotype calls
 134 (Figure 3A and C). This comparison assumes that the diploid calls are less affected by reference
 135 bias as slight deviations from a 50/50-ratio at heterozygous sites should be tolerated by a diploid
 136 genotype caller but random sampling would be biased towards the reference allele. This is sup-
 137 ported by the D statistic $D(chimp, reference; sf12_hapl, sf12_dipl) < 0$ ($Z = -13.5$), indicating
 138 more allele sharing between the reference and the pseudo-haploid calls. For this illustration, we
 139 are using diploid genotype calls from *GATK* as we are only looking at the variation at known SNP
 140 sites. We note that genotype callers specifically developed for ancient DNA (Link et al., 2017;
 141 Zhou et al., 2017; Prüfer, 2018) are preferable when calling novel variants from ancient DNA data
 142 as they incorporate post-mortem damage and other ancient DNA specific properties. Second, we
 143 compare randomly sampled reads of different fragment length categories (Figure 3B and D) as
 144 longer (75-80 bp) fragments should exhibit less reference bias than short (35-40 bp) fragments (see
 145 above), which is supported by the D statistic $D(chimp, reference; sf12_short, sf12_long) < 0$
 146 ($Z = -20.6$), indicating more allele sharing between the reference and pseudo-haploid calls from
 147 short fragments.

148 In general, we observe a deviation from zero in most cases highlighting the effect of reference bias
 149 on these statistics (Figure 3). Surprisingly, the directions of this bias differ between the HO data

150 and the SGDP data, which suggests that different reference data sets are also affected by reference
 151 bias at different degrees. This represents a potential batch effect which also needs to be considered
 152 when merging different reference data sets. Affinities to populations of different geographic origin
 153 vary in their sensitivity to reference bias but little general trends are observable. Western Eurasian
 154 populations show a strong deviation from 0 in all tests. Notably, African populations show the
 155 strongest deviation in the short versus long comparison in the SGDP data set while they exhibit
 156 almost no bias in the same comparison using the HO data. As the biases do not seem to show
 157 a consistent tendency, we cannot directly conclude that recent ancient DNA papers have been
 158 systematically biased in some direction. The shifts appear to be dataset and test specific so some
 159 results could still be driven by spurious affinities due to reference bias.

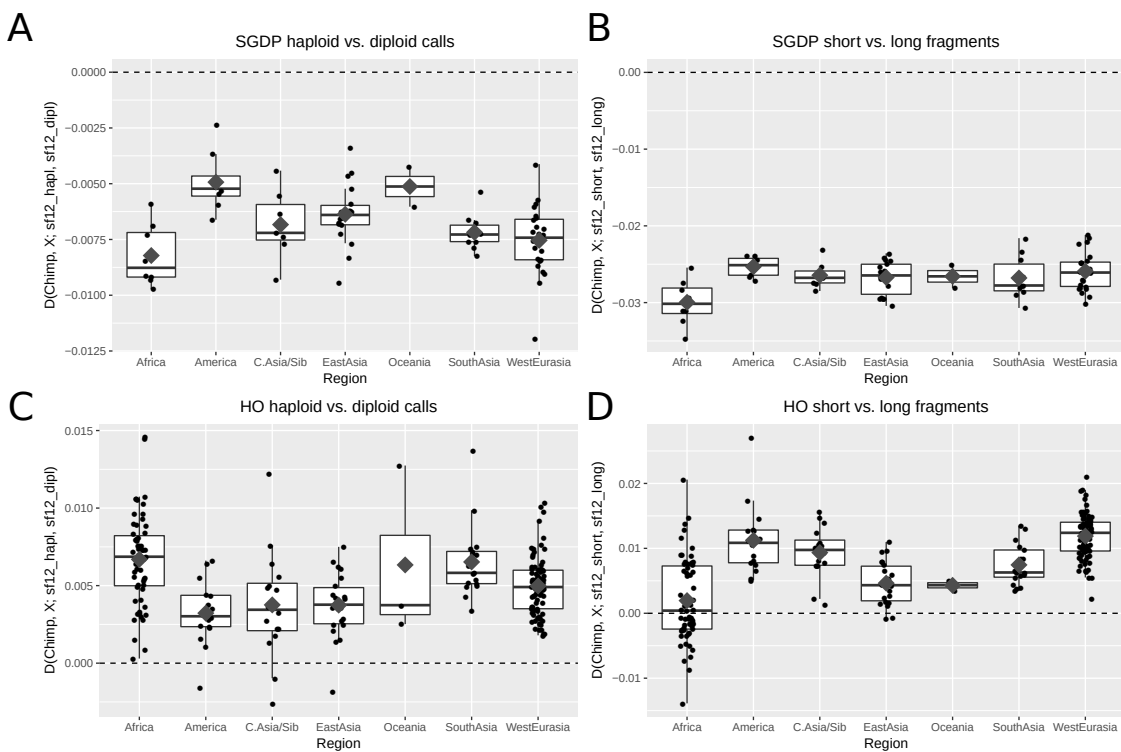


Figure 3: D statistics testing the affinity between different modern populations (X) and two different treatments of the high coverage individual $sf12$. The basis for these comparisons are the modern sequence data of the SGDP panel (A and B) or genotype data from the HO panel (C and D). Comparisons are done between pseudo-haploid and diploid calls for $sf12$ (A and C), and between pseudo-haploid calls from short (35-40 bp) or long (75-80 bp) fragments (B and D). The x axis represents the geographic origin of population X , diamonds show the mean for each continental group.

160 The human reference genome sequence is a mosaic of the genomes of different individuals. The
 161 geographic origin of the specific segments should have an impact on the population genetic affin-
 162 ties as the reference allele will more likely be found in specific geographic regions. We obtained
 163 information on the local ancestry of the human reference genome from Green et al. (2010). Ac-
 164 cording to this estimate 15.6 % of the reference genome can be assigned to African, 5.0 % to East

165 Asian and 30.0 % to European origin while the origin for 49.4 % is uncertain. We re-calculate
 166 D statistics for the different parts of the genome separately, restricting the analysis to the SGDP
 167 data. The impact of reference bias differs between the different ancestries (Figure 4). Generally,
 168 reference bias is weakest for reference segments of African origin. Notably, African populations
 169 show the strongest deviations from 0 in this case. Sequences mapping to the European segments of
 170 the reference show a strong reference bias with slight differences between continental populations.
 171 Reference bias at the East Asian segments of the reference genome seems intermediate but the D
 172 statistics also show large variation which may be due to the only small proportion of the reference
 173 genome that could confidently be assigned to an East Asian origin (Green et al., 2010).

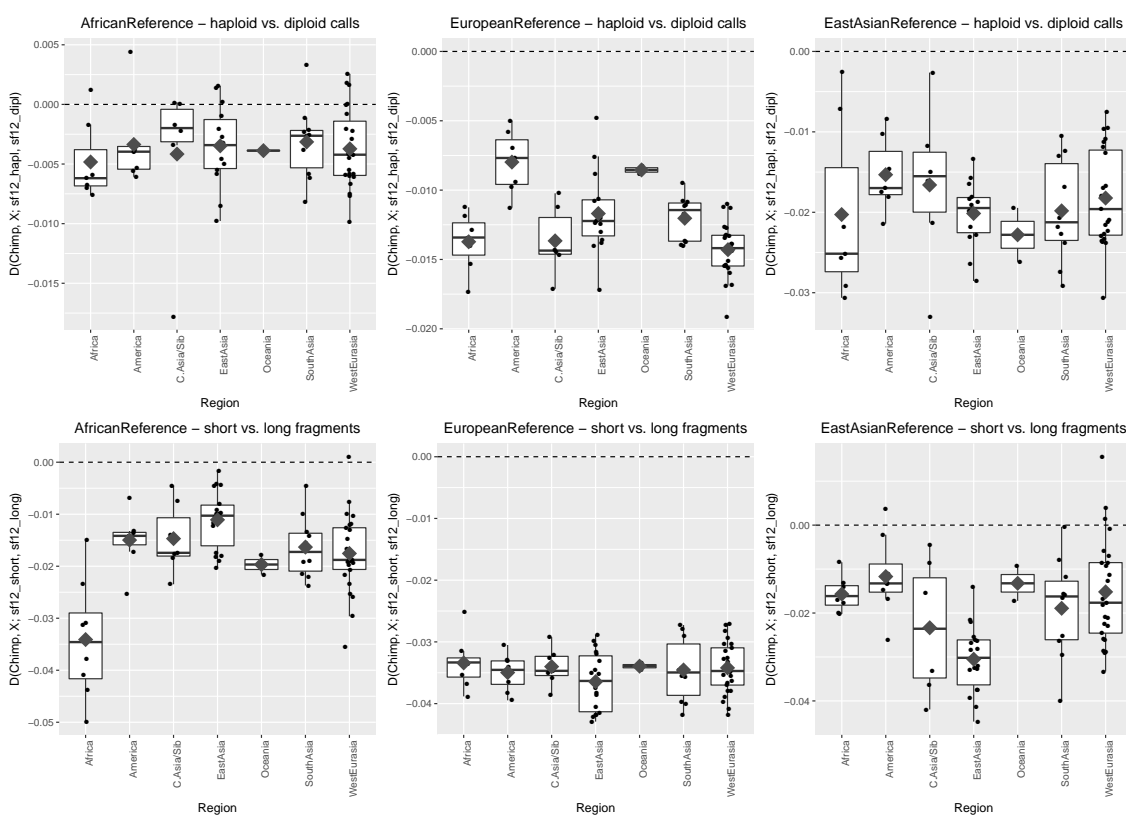


Figure 4: D statistics similar to Figure 3 for different parts of the reference genome depending on their geographic origin (Green et al., 2010). The x axis represents the geographic origin of population X , diamonds show the mean for each continental group.

174 Finally, we explore whether reference bias can affect estimates of archaic ancestry. We estimate
 175 the Neandertal ancestry proportion in sf12 as done by Prüfer et al. (2017):

$$\alpha = \frac{f_4(\text{sf12}, \text{Mbuti}; \text{AltaiNea}, \text{Chimp})}{f_4(\text{VindijaNea}, \text{Mbuti}; \text{AltaiNea}, \text{Chimp})}$$

176 We use eight different combinations of diploid and pseudo-haploid calls for sf12 as well as the
 177 two Neandertals in this statistic (Table 3). The 95% confidence intervals of all estimates overlap
 178 but point estimates differ by up to 1.25% when using all pseudo-haploid versus all diploid calls.

179 The African segments of the reference genome yield the lowest point estimates (as low as 1.42%)
 180 – some of these estimates are not even significantly different from 0. These differences highlight
 181 some of the sensitivities of f_4 -ratios not just to the choice of reference populations (Petr et al.,
 182 2018) but also to technical artifacts.

Table 3: Percentage of Neandertal ancestry (and standard errors) in sf12 using diploid and pseudo-haploid calls and different subsets of the human reference genome. Parts of the genome of East Asian origin were excluded due to their small total size.

Statistic [§]	Full reference	European Reference	African Reference
$f_4(sf12_h, Mbuti; AltaiNea_h, Chimp)$	3.15 ± 0.44	3.11 ± 0.80	2.47 ± 1.01
$f_4(VindijaNea_h, Mbuti; AltaiNea_h, Chimp)$	2.54 ± 0.44	2.70 ± 0.80	1.91 ± 1.01
$f_4(sf12_d, Mbuti; AltaiNea_d, Chimp)$	2.22 ± 0.43	2.76 ± 0.77	1.91 ± 0.98
$f_4(VindijaNea_d, Mbuti; AltaiNea_d, Chimp)$	2.79 ± 0.43	2.32 ± 0.76	1.42 ± 0.98
$f_4(sf12_h, Mbuti; AltaiNea_h, Chimp)$	2.68 ± 0.45	2.43 ± 0.80	2.59 ± 1.01
$f_4(VindijaNea_d, Mbuti; AltaiNea_h, Chimp)$	2.10 ± 0.44	2.07 ± 0.79	2.03 ± 1.00
$f_4(VindijaNea_d, Mbuti; AltaiNea_d, Chimp)$	2.45 ± 0.44	2.22 ± 0.77	2.12 ± 0.97
$f_4(sf12_d, Mbuti; AltaiNea_h, Chimp)$	1.90 ± 0.44	1.81 ± 0.76	1.63 ± 0.97
$f_4(VindijaNea_d, Mbuti; AltaiNea_d, Chimp)$			

[§] d and h denote diploid and pseudo haploid-calls, respectively

183 Potential data filtering strategies

184 After establishing the abundance and potential effect of reference bias, we investigated two simple
 185 post-mapping filtering approaches to mitigate reference bias. The two agents involved in the
 186 process are the reference genome and the sequence fragments or reads. We investigated 1,407,340
 187 of the SGDP transversion set of sites with at least 200 bp distance between two neighboring SNPs.
 188 First, we modified reads that successfully mapped to a SNP site with a match of the reference allele
 189 to carry the alternative allele. These modified reads were re-mapped to the reference genome and
 190 they passed the filtering if they still mapped to the same position of the genome with no indels.
 191 Second, we prepared a modified version of the reference genome which carried a third base (neither
 192 the reference base nor the known alternative allele) at all 1,407,340 sites. A similar approach has
 193 been used to study ultra-short fragments in sequence data from archaic hominins (de Filippo et al.,
 194 2018). All reads originally mapping to the SNP sites were re-mapped to this modified reference
 195 genome, and again only reads that mapped to the same location and without indels passed the
 196 filtering. Finally, we used both filters on the same BAM file. All scripts used for filtering can be
 197 found at <https://bitbucket.org/tguenther/refbias/>

198 The filtering approaches increase the average proportion of the alternative allele at homozygous
 199 sites (Figure 5A). Mapping to a modified reference genome shows a slightly better improvement
 200 than using modified reads, while combining both yields the best results in most cases. A small
 201 number of samples shows a 50/50-ratio after filtering but most are still significantly below that

202 ratio. This is not surprising as the filtering is only applied to reads that have previously mapped to
 203 a single reference genome so the data before filtering does not represent a 50/50-ratio, and removing
 204 some reference allele reads cannot completely account for the non-reference reads lost earlier. This
 205 is most evident in the data from Mathieson et al. (2015) which was only available as mapped
 206 reads after running *bwa* (Li and Durbin, 2009) with lower maximum edit distance parameters (-n
 207 0.04) than our pipeline which does not leave much room for improvement after filtering. Another
 208 possible reason for deviation from a 50/50-ratio at heterozygous sites could be low levels of modern
 209 contamination which may lead to a slight over-representation of the reference allele before mapping
 210 (Prüfer et al., 2014; Racimo et al., 2016; Prüfer, 2018). Comparing the outcome of the filters to
 211 different fragment length categories shows a similar pattern: the bias is decreased but some length
 212 categories still display differences in their relative heterozygosity (Figure 5B).

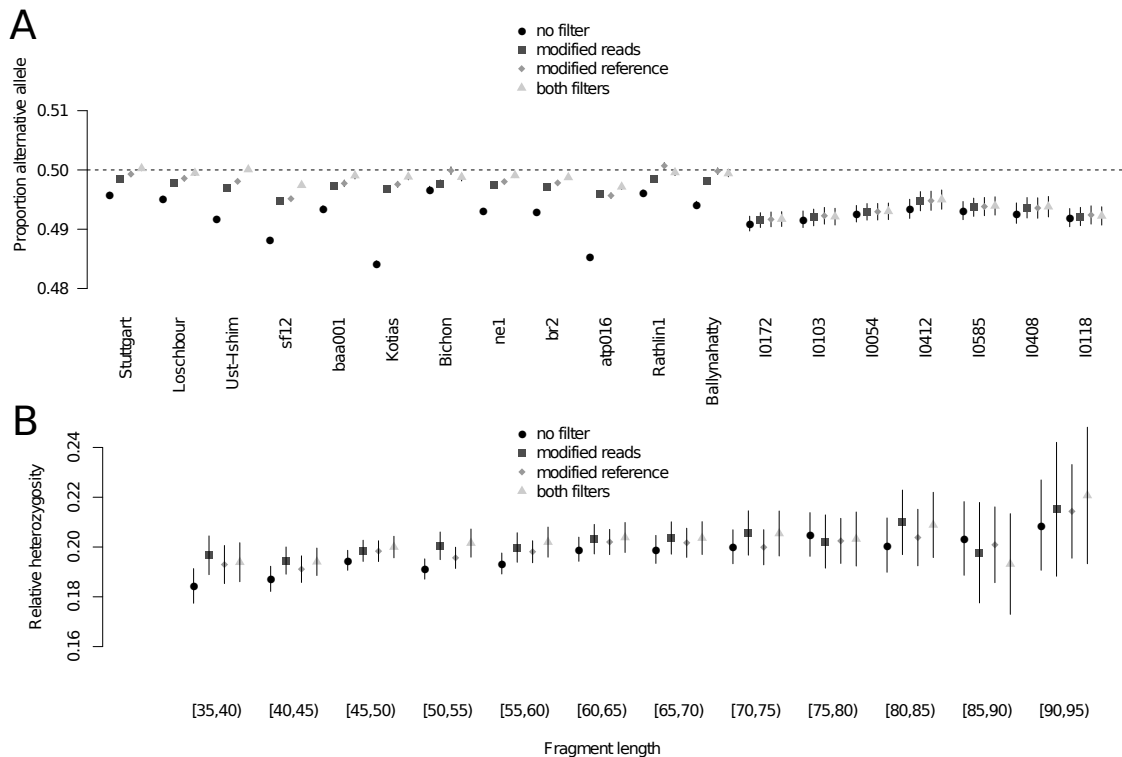


Figure 5: Comparison of different post-mapping filtering strategies for high coverage bam files from anatomically modern humans employing mapping and base quality filters of 30. (A) Average proportion of the alternative allele for the comparison between no additional filters (see also Figure 1), remapping of reads carrying the reference allele modified to carry the alternative allele (modified reads), remapping against a modified reference carrying a third allele at the SNP sites, and both filters together. (B) Influence of filtering on measures of heterozygosity for different fragment sizes in sf12. Error bars indicate two standard errors.

213 Discussion

214 Systematic biases are problematic in all types of quantitative research, and it is therefore important
215 to be aware of them and alleviate or avoid their effects as much as possible. Different systematic
216 biases in next-generation sequencing data have been investigated before (Prüfer et al., 2010; Ross
217 et al., 2013; Bobo et al., 2016; Ros-Freixedes et al., 2018), and it is known parameters such as
218 sequencing depth can influence population genomic estimates (Crawford and Lazzaro, 2012; Fu-
219 magalli, 2013; Korneliussen et al., 2013). Differences in sequencing strategies (e.g. read length) and
220 bioinformatic processing have been shown to generate batch effects and dramatically affect down-
221 stream analyses (Leek et al., 2010; Leigh et al., 2018; Shafer et al., 2016; Mafessoni et al., 2018).
222 Another well known bias in population genetics is ascertainment bias which arises when the studied
223 variants were ascertained in selected populations only, and can substantially impact measurements
224 of heterozygosity and related methods (Albrechtsen et al., 2010). The research community is aware
225 of these potential issues and they are avoided by filtering strategies, standardizing bioinformatic
226 pipelines, including controls and accounting for systematic biases in downstream analysis.

227 The common use of randomly sampled alleles and pseudo-haploid data in palaeogenomic re-
228 search can exacerbate the effect of reference bias compared to diploid genotype calls obtained from
229 medium to high coverage data. We show that reference bias is able to lead to significant differ-
230 ences between estimates of population genetic parameters (heterozygosity), overestimated levels
231 of archaic ancestry as well as to cause spurious affinities to certain populations. Mixing different
232 mapping parameters or minimum fragment lengths in the same study should generally be avoided.
233 Additionally, strong differences of fragment size distributions between different individuals may
234 cause spurious affinities due to reference bias. Many estimates from low coverage data are gener-
235 ally noisy, but studies show increasing sample sizes and amounts of data which means that subtle
236 biases become of increasing importance in the future. Notably, the bias for the whole genome
237 (Figure 3) seems less extreme than some of the results for ancestry-specific segments (Figure 4)
238 suggesting that the mosaic nature of the human reference genome may reduce the bias to some
239 degree as different regions will be biased in different directions. In this respect the human refer-
240 ence genome is different from many other species where the reference genome is derived from a
241 single individual which would increase the potential impact of reference bias on population genetic
242 analysis in other systems.

243 Our analysis does not directly indicate a strong direct impact of different wet lab procedures
244 on the observed average degree of reference bias. We caution, however, that such an effect may
245 exist as indicated by the correlations between samples processed in the same lab (Supplementary
246 Table 1). Different library preparation techniques produce different length distributions since
247 some approaches are directly targeting shorter fragments which will have an impact on mapping.

248 Furthermore, the SNP capture approaches used to generate the data we analyzed uses one bait
249 per allele minimizing reference bias before sequencing. Most whole genome or exome capture
250 approaches, however, are using baits designed from a single individual which should introduce a
251 pre-mapping bias towards the allele carried by that person (Quail et al., 2008; Heinrich et al., 2012;
252 Meynert et al., 2013; Lindo et al., 2016). Finally, contamination from another person should tend
253 to introduce the major allele which is likely the reference allele in most cases – a process that will
254 also increase reference bias before mapping (Prüfer et al., 2014; Racimo et al., 2016; Prüfer, 2018).

255 Our analysis of the distribution of reference bias across the genome for the sf12 individual
256 has several repercussions. First, most reads are neutral to changing the allele to its opposing
257 counterpart. This leads to a possible alternative filtering strategy. In cases where a pre-defined set
258 of variants is acceptable, a quality control should be performed on the study level to filter out SNPs
259 which correspond to reads that do not survive this alternative mapping. The exact details of such
260 a filter will, again, be dependent on the expected length and degradation of the reads. Another
261 important observation is that reference bias does not operate alone. There is also a weaker, but
262 very clear, signal of alternative allele bias, affecting roughly 0.6% of the total SNPs. In addition,
263 both reference and alternative bias can sometimes be very strong on the level of individual SNPs.
264 Even in a dataset with an overall proportion of alternative reads close to 0.5 in heterozygous sites
265 overall, subsets of SNPs might perform very differently, again possibly confusing deeper forms of
266 analysis that do not only consider genome-wide metrics – for example selection scans or analysis
267 of loci involved in certain traits.

268 We show, that filtering steps can reduce but not completely eliminate reference bias at SNPs
269 after mapping. To fully prevent reference bias, alternative mapping strategies would be needed
270 or filtering strategies would have to be developed for all raw data which is not always published.
271 Furthermore, these proposed filters require a pre-defined set of variants used for downstream anal-
272 ysis and are not suitable for calling novel variants from ancient DNA data. The latter, however,
273 will generally be only restricted to high quality and high coverage samples. A recently developed
274 genotype caller for ancient DNA data estimates reference bias from the data and uses the estimate
275 as a parameter for variant calling (Prüfer, 2018), which seems to work well for samples sequenced
276 to coverages of 15x or higher. One could use the filtering steps tested by us in a similar manner to
277 estimate what proportion of reads in a library are affected by reference bias which could later be
278 used to estimate genotype likelihoods (Nielsen et al., 2011; Wang et al., 2013). As reference bias
279 is somewhat predictable and detectable, this offers opportunities to account for it in downstream
280 analyses (e.g. Bryc et al., 2013; Wu et al., 2017).

281 Alternative mapping strategies such as mapping against genome graphs (Paten et al., 2017;
282 Garrison et al., 2018) or multiple reference genomes simultaneously (Schneeberger et al., 2009)

283 could be able to eliminate reference bias already in the mapping step. These approaches are not
284 broadly established in human genomics yet but their development has huge potential with regard
285 to reference bias. Such approaches could also lead to an increase in the total amount of authentic
286 data that can be obtained from a library while post-mapping filters will reduce the amount of data
287 used for downstream analyses (between 2 and 10 % in our cases). In addition to filtering data
288 and standardizing bioinformatic pipelines for all samples used in a study (both published data and
289 newly sequenced), we propose simulations as a potential control. Specific ancient DNA simulation
290 suites (Renaud et al., 2017) provide the opportunity to simulate data exactly matching fragment
291 size and damage patterns of empirical ancient DNA data so one can use them to study if observed
292 patterns may be driven by reference bias alone.

293 The present study focused mainly on humans but the effect of reference bias extends to other
294 species as well. The slight alternative bias in archaic hominins and the different population affinities
295 depending on the geographic origin of the reference genome illustrate that increasing evolutionary
296 distance can exacerbate reference bias or even cause systematic alternative bias at some sites.
297 This suggests that mapping against a reference genome of a related species (in the absence of a
298 reference genome for the species in focus) may impact downstream analyses as well (Green et al.,
299 2010; Schubert et al., 2012; Shapiro and Hofreiter, 2014; Gopalakrishnan et al., 2017), but the
300 population genetic bias may be weaker as the reference genome employed usually represents an
301 outgroup of equal distance to all individuals in the studied species.

302 Conclusion

303 Our analysis highlights that reference bias is pervasive in ancient DNA data used to study pre-
304 historic populations. While the strength of the effect differs between applications and data set,
305 it is clear that reference bias has the potential to create spurious results in population genomic
306 analyses. Furthermore, even when the overall presence of bias is limited, it is important to assess
307 whether subsets of variants are prone to strong systematic bias, including the possible presence of
308 alternative bias.

309 We are entering a time where sample sizes in ancient DNA studies reach one hundred and
310 beyond, while the questions focus on more and more detailed patterns and subtle differences. At
311 the same time, sampling starts to involve older remains and remains from more challenging en-
312 vironments – both of which are usually associated with poor preservation and shorter fragments.
313 Therefore it seems crucial to avoid reference bias or other biases such as batch effects or ascertain-
314 ment biases as much as possible, and to develop and apply computational strategies to mitigate
315 the impact of these issues.

316 **Materials and Methods**

317 **Data sets and bioinformatic processing**

318 We selected medium to high coverage data from 22 different individuals representing data generated
319 by different research groups with different wet lab strategies, covering different geographic regions
320 and time periods (Table 1). For anatomically modern human samples, we tried to use data as raw
321 as possible but some publications only provided the data after mapping and filtering. The general
322 pipeline for these samples was identical to previous studies (Günther et al., 2015, 2018). Reads
323 were mapped to the 1000 genomes version of the human reference genome hg19 using *bwa* (Li and
324 Durbin, 2009) with non-default parameters `-l 16500 -n 0.01 -o 2`. Subsequently, PCR duplicates
325 and fragments shorter than 35 bp were filtered (Kircher, 2012).

326 We restricted our analysis to a set of known transversion variants to avoid an effect of post-
327 mortem damage. We selected 107,404 transversions from the Human Origins panel (Patterson
328 et al., 2012; Lazaridis et al., 2014) as well as 1,693,337 transversions which were at at least 5%
329 allele frequency in the public data of the Simons Genome Diversity Project (SGDP, Mallick et al.,
330 2016). To detect reference bias, we are looking at supposedly heterozygous sites where one would
331 expect reads to map in a 50/50-ratio on average if no bias existed. We define a heterozygous site
332 as a SNP for which we observe at least ten reads with between 25 to 75% of those representing
333 the alternative allele. These reads are assessed using *samtools mpileup* (version 1.5, Li et al., 2009)
334 employing the `-B` option to turn off base quality rescaling.

335 For the high coverage genome of sf12 (Günther et al., 2018) as well as the high coverage archaic
336 genomes (Meyer et al., 2012; Prüfer et al., 2014, 2017) we also generated diploid genotype calls
337 following the pipeline described in Günther et al. (2018). Briefly, base qualities of all Ts in the
338 first five base pairs of each read as well as all As in the last five base pairs were set to 2. *Picard*
339 version 1.118 (Broad Institute, 2016) was used to add read groups to the files followed by indel
340 realignment with *GATK* 3.5.0 (McKenna et al., 2010) based on reference indels identified in phase
341 1 of the 1000 genomes project (Auton et al., 2015). Finally, diploid genotypes were called with
342 *GATK*'s UnifiedGenotyper employing the parameters `-stand_call_conf 50.0, -stand_emit_conf`
343 `50.0, -mbq 30, -contamination 0.02` and `-output_mode EMIT_ALL_SITES` using dbSNP version
344 142 as known SNPs. Genotype calls not flagged as low quality calls at investigated SNP sites were
345 extracted from the VCF files using *vcftools* (Danecek et al., 2011).

346 **Population genetic tests**

347 In order to investigate the population genetic effect of reference bias, we calculated D and f
348 statistics (Patterson et al., 2012). These statistics are based on pairwise allele sharing, so they

349 should be sensitive to spurious allele sharing due to reference bias. D statistics were calculated
350 with *popstats* (Skoglund et al., 2015), f_4 ratios were calculated *ADMIXTOOLS* (Patterson et al.,
351 2012), and standard errors were calculated employing a weighted block jackknife with a block size
352 of 5 Mbp. We used the chimpanzee reference genome as an outgroup.

353 Data Access

354 All scripts used for filtering can be found at <https://bitbucket.org/tguenther/refbias/>

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