Full Title:
Hybridization capture and low-coverage SNP profiling for extended kinship analysis and forensic identification of historical remains

Short Title:
Extended kinship analysis of historical remains using SNP capture

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Abstract

DNA-assisted identification of historical remains requires the genetic analysis of highly degraded DNA, along with a comparison to DNA from known relatives. This can be achieved by targeting single nucleotide polymorphisms (SNPs) using a hybridization capture and next-generation sequencing approach suitable for degraded skeletal samples. In the present study, two SNP capture panels were designed to target ~25,000 (25K) and ~95,000 (95K) autosomal SNPs, respectively, to enable distant kinship estimation (up to 4th degree relatives). Low-coverage SNP data were successfully recovered from 14 skeletal elements 75 years postmortem, with captured DNA having mean insert sizes ranging from 32-170 bp across the 14 samples. SNP comparison with DNA from known family references was performed in the Parabon Fχ Forensic Analysis Platform, which utilizes a likelihood approach for kinship prediction that was optimized for low-coverage sequencing data with DNA damage. The 25K and 95K panels produced 15,000 and 42,000 SNPs on average, respectively allowing for accurate kinship prediction in 17 and 19 of the 21 pairwise comparisons. Whole genome sequencing was not able to produce sufficient SNP data for accurate kinship prediction, demonstrating that hybridization capture is necessary for historical samples. This study provides the groundwork for the expansion of research involving compromised samples to include SNP hybridization capture.

Author Summary

Our study evaluates ancient DNA techniques involving SNP capture and Next-Generation Sequencing for use in forensic identification. We utilized bone samples from 14 sets of previously identified historical remains aged 70 years postmortem for low-coverage SNP genotyping and extended kinship analysis. We performed whole genome sequencing and hybridization capture with two SNP panels, one targeting ~25,000 SNPs and the other targeting ~95,000 SNPs, to assess SNP recovery and accuracy in kinship estimation. A genotype likelihood approach was utilized for SNP profiling of degraded DNA characterized by cytosine deamination typical of ancient and historical specimens. Family reference samples from known relatives up to 4th degree were genotyped using a SNP microarray. We then utilized the Parabon Fχ Forensic Analysis Platform to perform pairwise comparisons of all bone and reference samples for kinship prediction. The results showed that both capture panels facilitated accurate kinship prediction in more than 80% of the tested relationships without producing false positive matches (or adventitious hits), which were commonly observed in the whole genome sequencing comparisons. We demonstrate that SNP capture can be an effective method for genotyping of historical remains for distant kinship analysis with known relatives, which will support humanitarian efforts and forensic identification.

Introduction

Forensic laboratories are beginning to explore the use of large-scale single nucleotide polymorphism (SNP) panels for human identification in routine casework. The interrogation of a single-base target increases the likelihood of successful genotyping for degraded DNA samples, which is particularly beneficial for cases involving missing persons and historical remains with poor quality DNA. SNPs are known to have a low mutation rate making them valuable for distant kinship analysis (1), empowering kinship comparisons amongst distant relatives and facilitating the expansion of suitable references outside the immediate family members. This is of particular importance in decades-old cases, where generational gaps caused by deficient pedigrees and the passage of time may prevent closely-related references from being obtained.

Recent attention on SNPs in forensics has been primarily driven by the use of next-generation sequencing (NGS), which provides a means of generating massive amounts of data at one time for a large number of SNP loci. Commercially available NGS forensic kits, such as the ForenSeq DNA Signature Prep Kit (Verogen) and the Applied BioSystems Precision ID panels (Thermo Fisher Scientific), incorporate 100-200 SNPs for identity, phenotype and biogeographical ancestry inferences. Yet, the nature of DNA that can be recovered from severely compromised samples (e.g., fragmented, damaged, and low endogenous content) limits the applicability of these amplicon-based tools, and the small number of SNP markers impedes their use for distant kinship inference. To overcome these limitations of amplicon-based assays, Phillips *et al.* describe a custom SNP panel of ~1200 SNPs designed for the challenging samples encountered in missing persons efforts (2,3). The International Commission on Missing Persons is working

with QIAGEN (Hilden, Germany) to develop and validate this SNP panel as a QIAseq Targeted DNA Custom assay, which employs a single primer extension approach that is amenable to samples with reduced fragment length (<100 bp). This SNP kit will allow for direct comparisons between case samples and family references for distant kinship inference.

Alternatively, high-density genotyping arrays targeting up to a million or more SNPs have been used recently in cold cases to obtain genome-wide data from unknown crime scene samples for investigative genetic genealogy. Upload of these forensic profiles to publicly available genealogy DNA databases, such as GEDmatch (4) and FamilyTreeDNA (5), has facilitated new leads and identified suspects where other lines of investigation have been exhausted (e.g., (6-8)). SNP arrays have therefore provided the key to unlock genetic genealogy as a forensic investigative tool. However, while the SNP arrays utilized for genetic genealogy by direct-to-consumer (DTC) DNA testing companies have been used on a wide variety of forensic case samples, including bone (9), they require high quantities of intact human DNA (≥ 1 ng) and are sensitive to severe degradation and microbial contamination. Thus SNP arrays are not effective for highly degraded and/or environmentally challenged samples. For this reason, scientists are pursuing whole genome sequencing (WGS) as an alternative means to collect genome-wide SNP data from skeletal remains and other challenging samples. In one recently published study, WGS was utilized to generate SNP genotypes from unidentified human remains, providing information to help determine the identity of the unknown victim (10). The drawback to WGS is that it is expensive, especially when sequencing samples with a high percentage of microbial DNA that is co-extracted from the human bone, such as in historical cases and other unidentified skeletal remains (11). Scientists are now applying imputation methods (12-14) to produce complete genotypes from low coverage genomes in forensic cases (15).

Hybridization capture targeting the mitochondrial genome and small (~1200) SNP sets for identity testing has been successfully applied to forensic casework, including degraded and chemically treated samples (16-19). Yet due to the time and cost involved in hybridization capture, this method is not widely used in forensic casework, which can largely be solved using routine short tandem repeat (STR) typing or SNP arrays for genetic genealogy. Therefore, hybridization capture is, at the time of writing, most often reserved for the poorest quality samples or for those cases involving historical remains. Much broader use of hybridization capture is seen within the field of ancient DNA (aDNA). Such archaeogenomic research has shown the ability to obtain SNP genotypes from aDNA samples using hybridization capture and demonstrated that such data can be used to establish relationships between individuals and groups of individuals (20-22). However, the methods involved in aDNA studies are not easily implemented within regulated forensic laboratories. For example, the SNP probes may be made in-house and are not commercially available, or the software used to perform data analysis is not available to external users. Consequently, the gains made within the field of aDNA to advance SNP capture from skeletal remains are not directly transferrable to forensic practitioners.

116 The goal of this study was to obtain thousands of SNPs from previously identified historical
117 remains for kinship comparison to known references. Familial relationships ranging from
118 parent/offspring to 4th degree relatives were represented in the 14 cases. The generation of SNP
119 profiles used for this study was achieved via three methods: WGS and two levels of targeted
120 SNP capture (~25,000 SNPs and ~95,000 SNPs). Kinship analysis was completed using the
121 Parabon Fχ Forensic Analysis Platform (Parabon NanoLabs; Reston, Virginia) (23). An
122 evaluation of the efficacy of the three SNP approaches to achieve strong kinship support was
123 performed, which would ultimately aid in the identification of unidentified human remains.
124
125 **Materials and Methods**
126 Non-probative casework samples
127 *Sample selection*
128 Fourteen previously identified, non-probative skeletal samples from the Armed Forces Medical
129 Examiner System's Armed Forces DNA Identification Laboratory (AFMES-AFDIL) were
130 selected for use in this study (Table 1). These specimens originated from a variety of case
131 contexts, and all were approximately 70 years postmortem. Samples were selected based on the
132 availability of family reference samples (FRS) for kinship comparison.
133

134 **Table 1.** Sample information and laboratory processing procedures for 14 non-probative
135 casework samples used in this study. Relationship information for 22 family reference samples
136 (FRS) is also listed. Non-probative samples are divided into two sample sets for laboratory
137 processing and sequencing. An example pedigree demonstrating familial relationships is
138 presented in Figure S1.

Sample Set	Sample #	Specimen Type	DNA Extraction Method	Associated FRS	Relationship to Unknown	Degree of Relatedness
1	1	Long bone	Organic	1-A	First cousin once removed	4th degree
				1-B	First cousin once removed	4th degree
	2	Right humerus	Organic	2-A	Grand-nephew	3rd degree
				2-B	Grand-niece	3rd degree
	3	Right humerus	Organic	3-A	Niece	2nd degree
				3-B	Niece	2nd degree
				3-C	Nephew	2nd degree
				3-D	Nephew	2nd degree
	4	Right tibia	Organic	4-A	Son	Parent/offspring
	5	Right femur	Organic	5-A	Nephew	2nd degree
				5-B	Niece	2nd degree
	6	Right tibia	Organic	6-A	Sister	Full siblings
				6-B	Niece	2nd degree
2	7	Right femur	Organic/non-organic combined	7-A	First cousin once removed	4th degree
	8	Right femur	Organic	8-A	Grand-niece	3rd degree
	9	Left femur	Organic	9-A	Grand-niece	3rd degree
	10	Left femur	Non-organic	10-A	Grand-nephew	3rd degree
	11	Left femur	Organic	11-A	Sister	Full siblings
	12	Femur	Non-organic	12-A	Sister	Full siblings
	13	Tibia	Organic	13-A	Niece	2nd degree
	14	Tibia	Organic	14-A	Daughter	Parent/offspring
				14-B	Nephew	2nd degree

139
140
141 *DNA extraction and repair*
142 DNA was extracted from fragments of bone ranging from 0.2-1.0 g. Samples were first
143 powdered and demineralized overnight at 56°C in a buffer containing 0.5 M EDTA, 1%
144 lauroylsarcosine and 100 µl of 20 mg/ml proteinase K. DNA purification was achieved using
145 either an organic protocol or the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany)
146 as described in (24). One extraction control (reagent blank; RB) was included in each set (RB1
147 for set 1 and RB2 for set 2). In one instance (sample 7), previously generated DNA extracts were

148 combined prior to DNA repair due to low volumes in order to maximize the DNA input for
149 downstream processing.
150
151 A DNA repair step was performed on all DNA extracts, including RBs, using the NEBNext
152 FFPE DNA Repair Mix (New England BioLabs Inc, NEB; Ipswich MA). Purification of the
153 repaired DNA extract was performed using the QIAGEN MinElute PCR Purification Kit,
154 followed by elution in sterile Tris-EDTA [10 mM Tris, (pH 7.5) 0.1 mM EDTA]. DNA was
155 quantified with the dsDNA High Sensitivity (HS) Assay Kit on the Qubit 2.0 or Qubit 3.0
156 Fluorometers (all Thermo Fisher Scientific, Waltham, MA) to determine input into NGS library
157 preparation.
158
159 *Library preparation*
160 The KAPA Hyper Prep Kit (Roche Sequencing, Pleasanton, CA) was used for library
161 preparation. To evaluate the success of the library and capture procedures, a positive control
162 (PC) was initiated at the beginning of library preparation for each sample set (PC1 for set 1 and
163 PC2 for set 2). Adapter ligation was completed using dual-indexed adapters (Integrated DNA
164 Technologies, Coralville, IA) matching sequences used in the Illumina TruSeq HT kits at an
165 adapter concentration of 15 µM. Ten cycles of PCR amplification were completed on a
166 GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) following
167 the manufacturer's recommendations. Samples were eluted in 25 µl of Tris-EDTA following
168 bead-based cleanup. Libraries from all sample sets were quantified on the 2100 BioAnalyzer
169 instrument with the Agilent DNA 7500 Kit (both Agilent Technologies, Santa Clara, CA).
170
171 *25K and 95K SNP capture panels*
172 Two custom SNP panels were designed to allow overlap with the Infinium CytoSNP-850K v1.1
173 BeadChip (Illumina, San Diego, CA) for extended kinship comparisons. The first panel
174 contained 24,999 genomic SNPs (Table S1; referred to as the 25K SNP panel) and the second
175 panel included 94,752 genomic SNPs (Table S2; referred to as the 95K SNP panel). A majority
176 of the SNPs chosen were bi-allelic, and while not specifically targeted for their polymorphic
177 status, smaller proportions were tri- and tetra-allelic (Table S3). Criteria for SNP target selection
178 included likelihood to perform well for kinship (i.e. high minor allele frequency in all
179 populations, low F_{ST}, and low linkage disequilibrium between SNPs), distribution across the
180 genome, and the GC content of the surrounding genomic region (25-60%). The presence of
181 candidate SNPs in commercially available microarray kits ensured kinship comparison could be
182 performed with samples genotyped using microarray platforms (i.e. high quality FRS).
183
184 *Hybridization capture of SNP panel targets*
185 Hybridization capture of the 25K and 95K SNP panel targets was facilitated by myBaits Custom
186 DNA-Seq kits (Arbor Biosciences, Ann Arbor, MI). Bait design for the 25K panel consisted of
187 four baits per SNP (100,000 unique RNA baits) and the 95K SNP panel was designed with one

188 or two baits per SNP (180,000 unique RNA baits). The hybridization conditions followed the
189 manufacturer's recommended protocol (25) with a 24-hour hybridization at 65°C using a Veriti
190 thermal cycler (Thermo Fisher Scientific). An attempt was made to target the maximum
191 recommended DNA input into capture (500 ng). For some samples, capture input was reduced
192 for the 95K panel due to lack of remaining library (Table S4). The captured product was split
193 into two portions and both portions were amplified independently for 19 PCR cycles with KAPA
194 HiFi HotStart ReadyMix (Roche Sequencing). The two amplified capture products for each
195 sample were combined and purified with the MinElute PCR Purification Kit, eluting in 25 µl of
196 Tris-EDTA. Purified capture product was quantified using the 2100 BioAnalyzer instrument with
197 the Agilent DNA 7500 Kit.
198
199 *Library pooling and sequencing*
200 Each sample set was sequenced separately by pooling an equal volume of captured libraries
201 within the set (excluding the PC). The PCs were not sequenced, as previous studies have shown
202 sequencing of PCs with low quality samples can cause instrument crosstalk and complicate data
203 interpretation (17,26,27). Pool molarity was determined using the Agilent DNA 7500 Kit on the
204 2100 BioAnalyzer instrument. Pools were loaded for sequencing at 10 pM and spiked with PhiX
205 Sequencing Control v3 (Illumina) at a 2.5% concentration. Sequencing was performed on a
206 MiSeq FGx Forensic Genomic System (Verogen, San Diego, CA) in Research Use Only mode.
207 Paired-end sequencing was completed using 300-cycle MiSeq Reagent Kits v2 (Illumina; 2 x
208 150 cycles).
209
210 WGS was also performed using the same libraries as those enriched using hybridization capture.
211 Libraries for WGS were individually normalized to 2 nM and pooled in equimolar concentration.
212 For the MiSeq runs, the pools were diluted to 10 pM and prepared for sequencing with 2.5%
213 PhiX as described above. Paired-end sequencing was completed using MiSeq v2 Reagent Kits
214 (300-cycle; 2 x 150) on a MiSeq FGx in Research Use Only mode.
215
216 Four libraries (95K capture products and WGS libraries for both samples 2 and 5) were
217 sequenced using the Illumina NextSeq 550 in two sequencing runs. Pools were generated as
218 described for the 95K and WGS libraries. The pools were loaded at 1.45 pM (80% of the
219 manufacturer's 1.8 pM recommended loading concentration) with 1% PhiX control spiked-in as
220 a sequencing control. Paired-end sequencing was completed using NextSeq 500/550 300-cycle
221 High-Output Kits v 2.5 (Illumina; 2 x 150 cycles). High-Output kits sequenced on the NextSeq
222 allow for over 25 times more sequence data to be generated compared to MiSeq v2 sequencing
223 (800M versus 30M paired reads, respectively).
224
225
226
227

228 *Microarray genotyping*
229 DNA extracts from two additional samples were sent to AKESOgen (Peachtree Corners, GA), a
230 CLIA-certified sequencing laboratory, for microarray genotyping with the Illumina Infinium
231 CytoSNP-850K v1.1 BeadChip. Both unrepaired and repaired DNA extracts were tested using
232 two genotyping protocols. Genotyping was halted after the first four samples failed to generate
233 any usable data (genotyping call rate <50%). A summary of these results is available in Table S5,
234 but no further discussion will be provided below due to the poor quality of the genotyping data.
235
236 Family reference samples
237 *Sample selection and DNA extraction*
238 A total of 22 FRS were selected for use in this study based on known relationships to the
239 previously identified case samples (Table 1). An additional 17 FRS with no relation to the case
240 samples were also genotyped, totaling 39 FRS for comparison. All FRS donors provided
241 informed consent for samples to be used in research and quality improvement activities.
242 Reference specimens were saliva samples deposited on either Bode buccal collectors or cotton
243 buccal swabs. The QIAamp DNA Investigator Kit (QIAGEN) was employed for DNA extraction
244 following one of two manufacturer protocols: isolation of total DNA from FTA and Guthrie
245 cards using two punches from Bode buccal collectors, or isolation of total DNA from surface and
246 buccal swabs using one buccal swab. The final elution volume for all samples was 100 µl of
247 Tris-EDTA. DNA was quantified using both the Qubit dsDNA HS Assay Kit on the Qubit 3.0
248 Fluorometer and the Plexor HY DNA Quantification Kit (Promega Corporation, Madison, WI)
249 prior to submission for microarray genotyping.
250
251 *Microarray genotyping*
252 FRS were genotyped at AKESOgen using the Infinium CytoSNP-850K v1.1 BeadChip.
253 Genotypes were called using the GenomeStudio Software (Illumina) and the data were reviewed
254 prior uploaded to the Parabon Fχ Forensic Analysis Platform for analysis.
255
256 Data analysis
257 *Non-probative case samples*
258 Demultiplexed FASTQ files were generated from the raw data by the MiSeq Reporter software
259 (Illumina) and imported into the Parabon Fχ Forensic Analysis Platform. Reads were aligned to
260 the human reference genome (GRCh38), and duplicate mapped reads were removed. Alignment
261 parameters included penalties for mismatches (4), open gaps (6), extension gaps (1) and unpaired
262 reads (9). Minimum map and base qualities were not required (i.e. set to 0) in order to allow for
263 the maximum number of called SNPs, which are assessed using a probabilistic approach.
264 Specifically, the software implements a genotype likelihood approach for each locus under
265 investigation, which facilitates the use of low coverage SNPs (≥ 1X) and maximizes the use of
266 available information (28). In short, the likelihood of the data under the hypothesis of each
267 possible genotype is calculated given factors such as the number of reads per allele and

268 sequencing quality score. This approach increases the probative power of the analysis and avoids
269 potentially erroneous genotype calls generated using an absolute calling threshold. To account
270 for the effects of DNA damage typical of ancient and historical remains, cytosine deamination
271 was assessed at the time of alignment (29,30). This assessment is performed utilizing a different
272 mapping (than the original alignment described above) as the read pairs are merged and aligned
273 to the GRCh38 as single-end reads. This allows for estimation of the mismatch rate at the end of
274 the fragments, specifically cytosine to thymine error on the 5' end and guanine to adenine error
275 on the 3' end, which are indicative of cytosine deamination (29,30). Based upon this assessment
276 (e.g., Figure S2), the user has the option to correct for the observed damage. In addition to the
277 use of the merged-pair mapping, the position within the fragment is incorporated into the
278 likelihood calculations when generating the SNP profile when the damage correction option is
279 selected. SNP genotype likelihood profiles were generated for each captured SNP set using read
280 depth thresholds of 1X, 5X and 10X. Though no enrichment was performed, WGS data were
281 analyzed for the 843,223 SNPs present on the Infinium CytoSNP-850K BeadChip at the 1X, 5X
282 and 10X thresholds. All SNP genotype likelihood profiles were generated with and without
283 damage correction.
284
285 Ancestry inference was performed in Fχ for both the unknown and reference samples, which was
286 then used along with self-reported race (for the FRS donors) to determine the appropriate
287 population allele frequency file for kinship calculations. Due to the large number of SNPs
288 recovered in the FRS microarray data, ancestry prediction was possible for seven well-defined
289 global population groups (31). Five populations are represented in the ancestry prediction for the
290 sequenced (WGS and captured) samples, based on the 1000 Genomes Project (32). The Parabon
291 Fχ Forensic Analysis Platform estimates pairwise relatedness using the likelihood formulas
292 derived in (33), adapted for the scenario of testing a specific set of kinship hypotheses by
293 comparing genotype likelihoods to called genotypes from a reference sample. This approach
294 takes into account all possible genotypes for the captured sample, as well as the genotype
295 frequencies of the selected population. Kinship prediction was performed against the entire FRS
296 dataset (Figure S3), and log likelihoods were calculated within the software for each degree of
297 relatedness (DOR) up to 4th degree (from self/monozygotic twin, parent-offspring, full sibling,
298 2nd degree, 3rd degree, and 4th degree) as well as unrelated. Likelihood ratios (LRs) were
299 generated within the software for the most likely DOR compared to the unrelated category. LRs
300 over 10,000 were considered strong evidence of relatedness, based on previously established
301 forensic guidelines (34).
302
303 *Family reference samples*
304 FRS SNP genotypes were evaluated for genotyping call rate (i.e. the proportion of SNPs with
305 called genotypes), and those below 70% were removed from further analysis. The remaining
306 FRS SNP genotypes were loaded into the Parabon Fχ software, and each sample's ancestry

307 proportions were estimated. In addition to kinship comparisons with the case samples, kinship
308 between each FRS pair was predicted and compared to the expected relationships.
309
310 **Results**
311 *Non-probative casework sample assessment*
312 A total of 16 libraries were prepared and sequenced: 14 bone samples and two RBs. Sequence
313 data for all three SNP approaches (i.e. WGS, 25K, and 95K) were generated from each of the 16
314 libraries. Metrics for each of the nine sequencing runs can be found in Table S6, and alignment
315 and mapping metrics for all samples are presented in Table S7. Average DNA fragment length
316 (or mean insert size in Table S7) was only 40 bp in the WGS data, ranging from 20-140 bp
317 across the 14 case samples (Figure 1). However, the mean insert size after capture was
318 considerably larger, averaging ~120 bp in the 25K and 95K data. The reason for this discrepancy
319 is likely due to non-specific mapping of off-target, short reads between 20-25 bp. These short
320 reads are visible in the fragment length distributions as high peaks that break with the unimodal
321 distribution of the remaining mapped DNA that is larger in size (Figure 2). Typically, reads <30
322 bp are excluded from sequence alignments in aDNA research (e.g., (35)), which minimizes the
323 proportion of off-target read mapping. Such short read filtering may also be required for forensic
324 casework involving degraded DNA, and this parameter can be incorporated into future versions
325 of the Parabon Fχ software. The observed short read peaks are much more pronounced in the
326 WGS data than in the capture data, likely due to the effect of targeted enrichment on the
327 proportion of off-target reads causing the non-specific mapping. Therefore the mean insert size
328 indicated in the capture data is likely more accurate to the authentic human DNA present in the
329 case samples, with an average of ~120 bp. The capture data furthermore show that the fragment
330 length distributions vary by sample, as sample 3 (Figure 2a) has a maximum fragment length
331 exceeding 400 bp, while sample 11 (Figure 2c) contains endogenous human DNA that is
332 altogether <100 bp in length. Despite the skewed fragment length distribution due to the non-
333 specific mapping of off-target short reads, particularly in the WGS data, these samples were
334 shown to exhibit considerable DNA degradation consistent with expectations from ancient and
335 historical remains.
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337
338
339

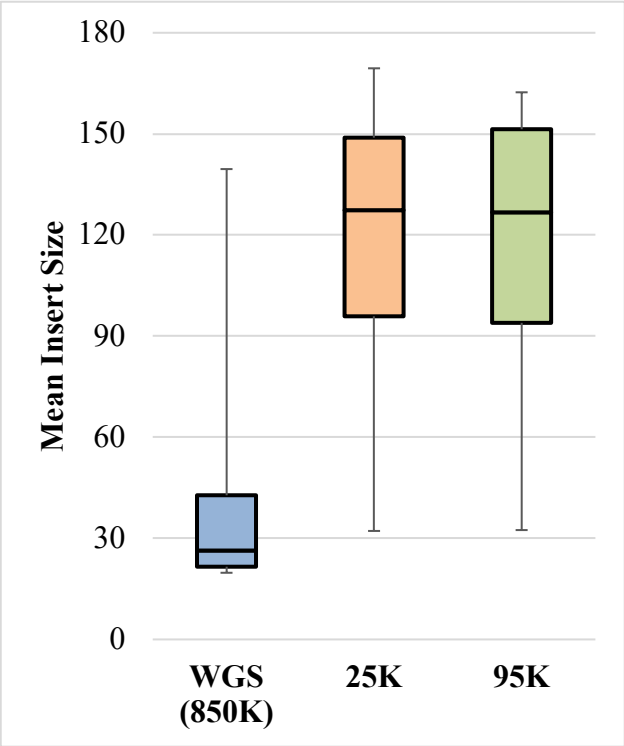
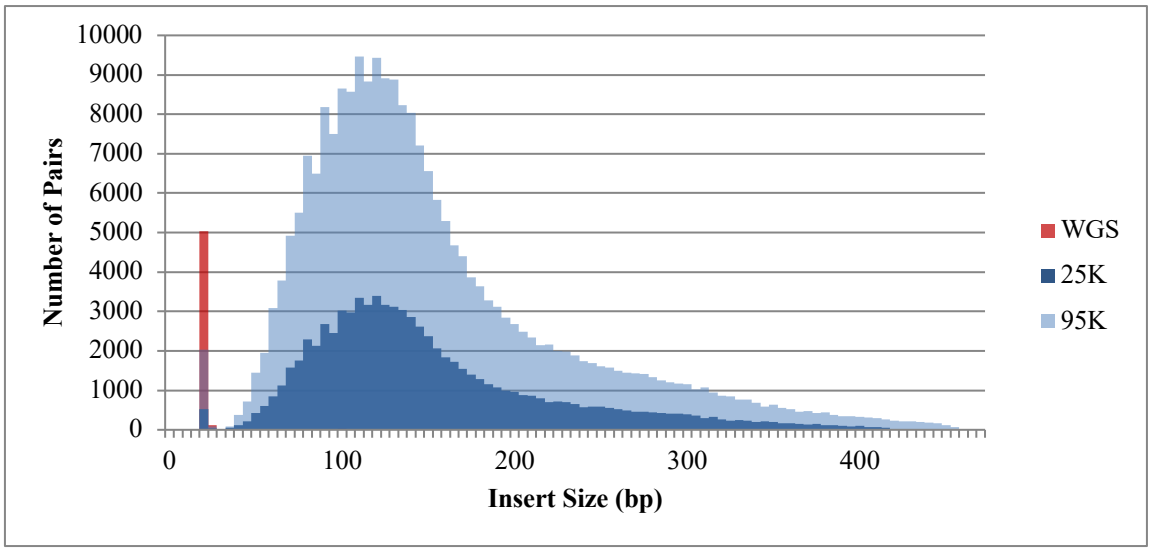
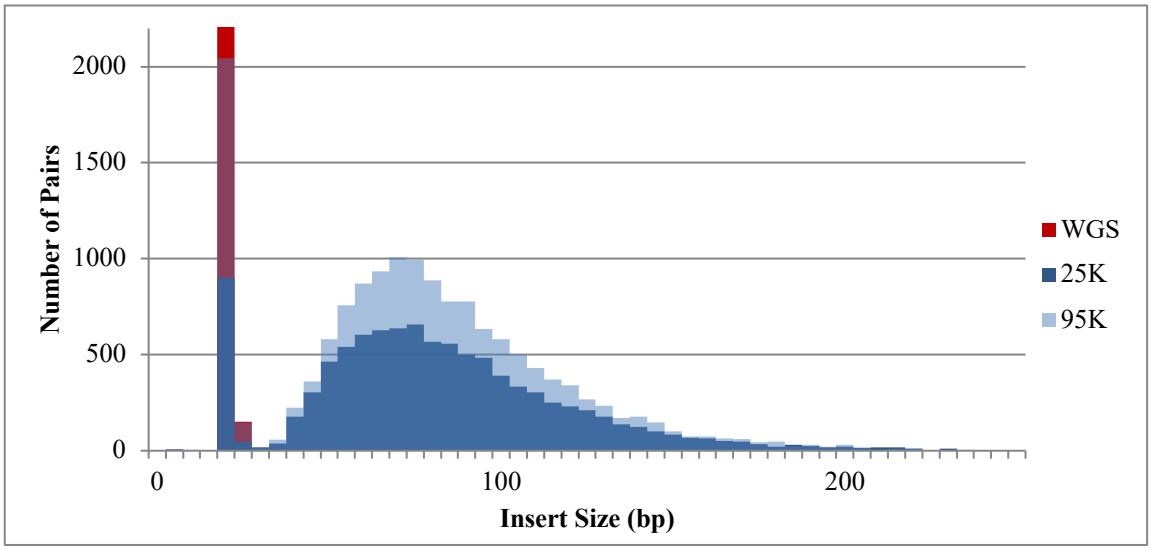


Figure 1. Distribution of the mean insert size of reads mapped to the human genome (GRCh38) across the three SNP sequencing approaches: whole genome sequencing (WGS), 25K SNP capture, and 95K SNP capture.

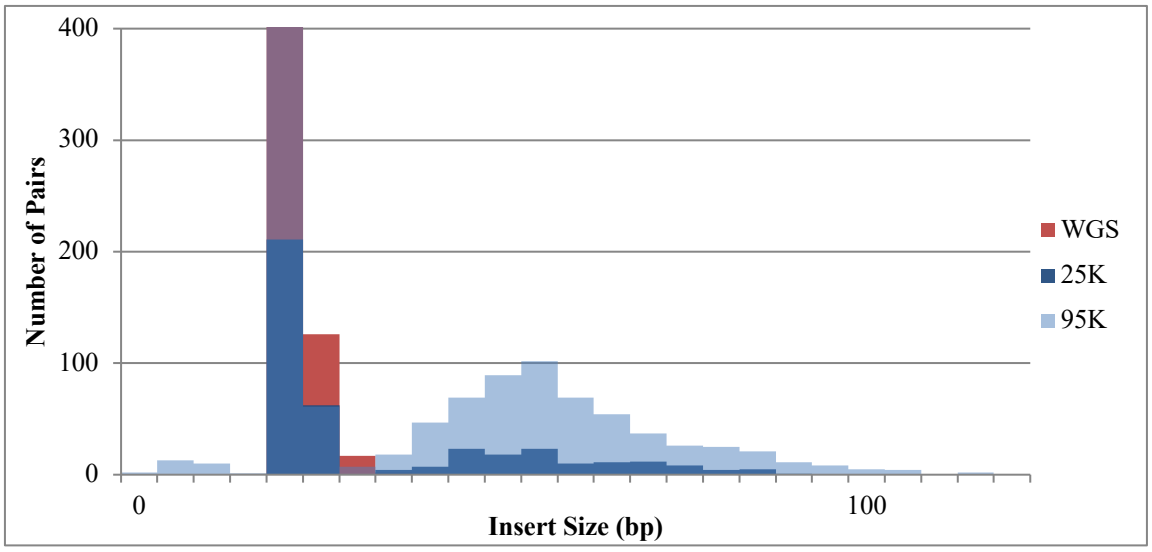
345



346 a.



347 b.



348 c.

Figure 2. Fragment length (insert size) distribution for three exemplary samples using whole genome sequencing (WGS), 25K and 95K capture. Samples represent the range of quality observed in this study: a) sample 3, b) sample 1, and c) sample 11. Note the difference in scale between samples for both the x and y axes. Peaks at 20-25 bp are likely due to non-specific mapping of off-target reads.

Despite the inclusion of short, off-target reads in the mapped sequence data that considerably impacted the WGS results by inflating the mapped proportion, the percent of total reads mapped to GRCh38 still increased after capture (25K or 95K) (Figure 3). Therefore capture increased the percent of endogenous human DNA in the library, indicating successful enrichment of the human SNP targets. Regardless of the improvement in endogenous DNA content, the proportion of human DNA in the library after capture remained less than 50% for a majority of the samples (8 out of 14). In other words, even after capture to enrich for human SNPs, many of the historical bone samples tested here contained more environmental DNA than human DNA in the sequenced libraries. To increase the endogenous human DNA proportion, a second round of capture can be performed. However, this adds time and expense to the procedure and should be considered on a case-by-case basis.

The generally poor quality of the samples tested in this study is typical of specimens submitted to the AFMES-AFDIL for DNA testing (11,17,36), and likely explains the failed microarray testing. Though other, higher quality forensic casework samples may be amenable to microarray testing (9), severely compromised samples such as those from decades-old missing persons cases may necessitate alternative genotyping methods for SNP recovery such as hybridization capture.

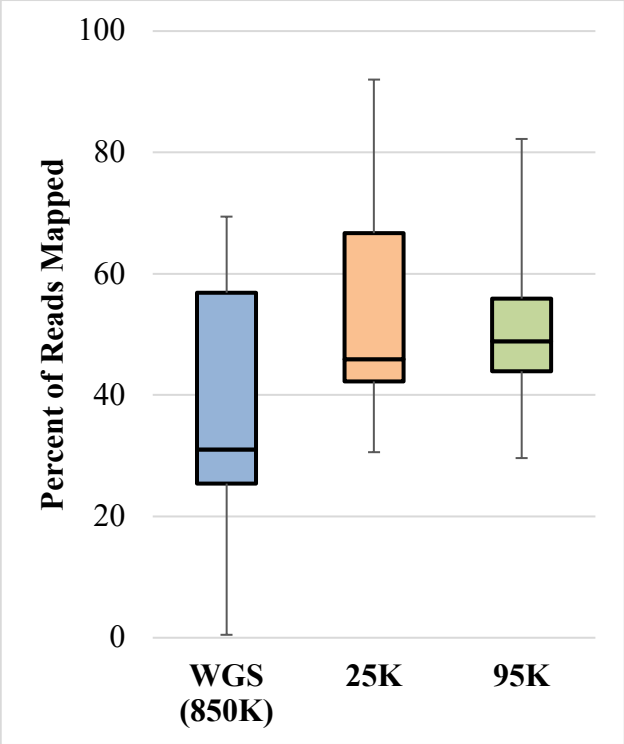


Figure 3. Distribution of the percent of reads mapped to the human genome (GRCh38) across the three SNP sequencing approaches: whole genome sequencing (WGS), 25K SNP capture, and 95K SNP capture.

Overall, WGS on the MiSeq was unable to produce adequate coverage of the "targeted" CytoSNP-850K BeadChip SNPs. The percentage of *unique* reads mapped to the human genome in the WGS data averaged 38.5%, but it ranged considerably between samples (0.49%-68.7%). Sample 14 was an obvious outlier, with only 0.49% of the unique reads mapped to the human genome, whereas the next-lowest sample had 22.9% unique reads that mapped to the human genome. Thus, degree of clonality (i.e. quantity of PCR duplicates) in each sequenced library varied by sample. Despite high read counts with an average of over 1.4 million unique reads in the MiSeq WGS data, only ~11,000 of these reads on average covered the ~850,000 SNP targets investigated (0.32%). Approximately 4,000 SNPs on average were covered in each sample at a 1X threshold (0.47%; Figure 4). SNP recovery after WGS on the MiSeq further decreased when damage correction was applied, leaving only one sample with >1,000 SNPs covered (sample 9). The two WGS libraries sequenced on the NextSeq generated significantly more data than those sequenced on the MiSeq. Over 1.1 million WGS reads covered the CytoSNP-850K BeadChip SNPs, and more than 230,000 SNPs were reported for each of the samples sequenced on this higher throughput sequencing platform (Table S7). It is important to note, however, that these samples (2 and 5) represent two of the best performing samples tested in this study. Since these two libraries lacked MiSeq WGS data for comparison, the percent increase in SNP yield when

398 going from a lower throughput to higher throughput sequencer cannot be determined.
399 Regardless, the WGS data, when produced either from a MiSeq or a NextSeq instrument, were
400 insufficient for accurate kinship prediction (discussed below).
401
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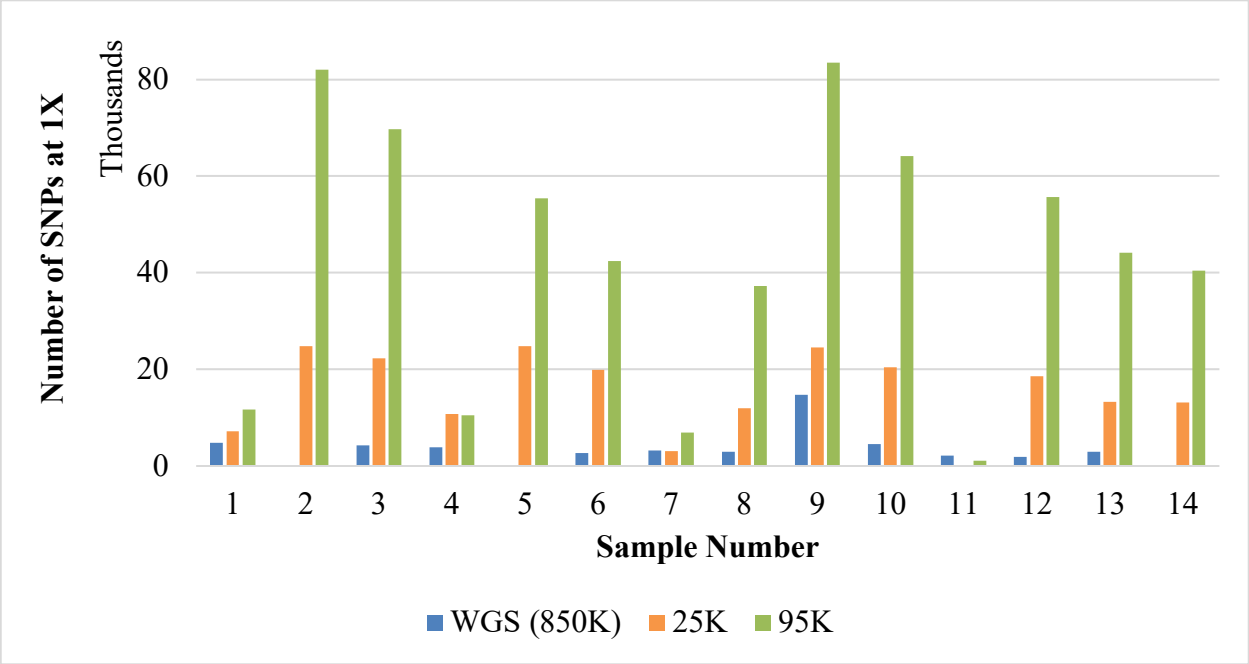

403
404 **Figure 4.** Number of SNPs recovered at ≥1X when 14 non-probative sample libraries were
405 sequenced on the MiSeq. WGS libraries for samples 2 and 5 were only sequenced on the
406 NextSeq and are not shown due to scale (281,346 SNPs and 230,616 SNPs, respectively).
407
408
409 Nearly all samples demonstrated an increase in the proportion of reads mapped to targeted SNPs
410 when hybridization capture was performed. The capture data resulted in an average of 4.1% and
411 4.5% of unique reads covering targeted SNPs in the 25K and 95K panels for an average of over
412 15,000 and 43,000 SNPs, respectively. Though low, the percentage of unique reads on target in
413 the capture data represent more than a 12-fold increase from the WGS data (0.3%). On an
414 individual sample basis, the SNP capture panels outperformed WGS in regards to overall SNP
415 recovery in all but one sample (sample 11, Figure 4). Sample 11 consistently performed poorly
416 after capture using both panels, generating 159 SNPs from the 25K panel and 625 SNPs from the
417 95K panel. Presumably, the relative failure of sample 11 is a consequence of the high degree of
418 DNA degradation present as the mean insert size was only 32 bp in the capture data, the smallest
419 of all the samples tested (Figure 2c; Table S7).
420
421 When comparing the performance of the 25K and the 95K panels, the raw number of SNPs
422 recovered was greater using the 95K panel in 13 of 14 samples (92.9%). Moreover, ten of the 14
423 samples (71.4%) produced more than 25,000 SNPs when the 95K assay was used for capture,

which is the maximum number of SNPs that could be achieved with the smaller 25K panel. One sample (4) produced fewer SNPs using the 95K capture assay when compared to the 25K capture assay; however, this can be attributed to the reduced DNA input for the 95K capture reaction due to insufficient remaining library volume, rather than panel performance. It is expected that had the maximum library input into capture been attained, increased SNP recovery would have been observed.

When the coverage threshold was increased from 1X to 5X, the average number of SNPs decreased by more than 60% in the 25K dataset and by 75% in the 95K dataset (Figure 5). When the coverage threshold was increased to 10X, the usable data were decreased by approximately 80-90% in both SNP panels, to approximately 3,000 SNPs. Few, if any, SNPs were obtained in the WGS data when the coverage threshold exceeded 1X.

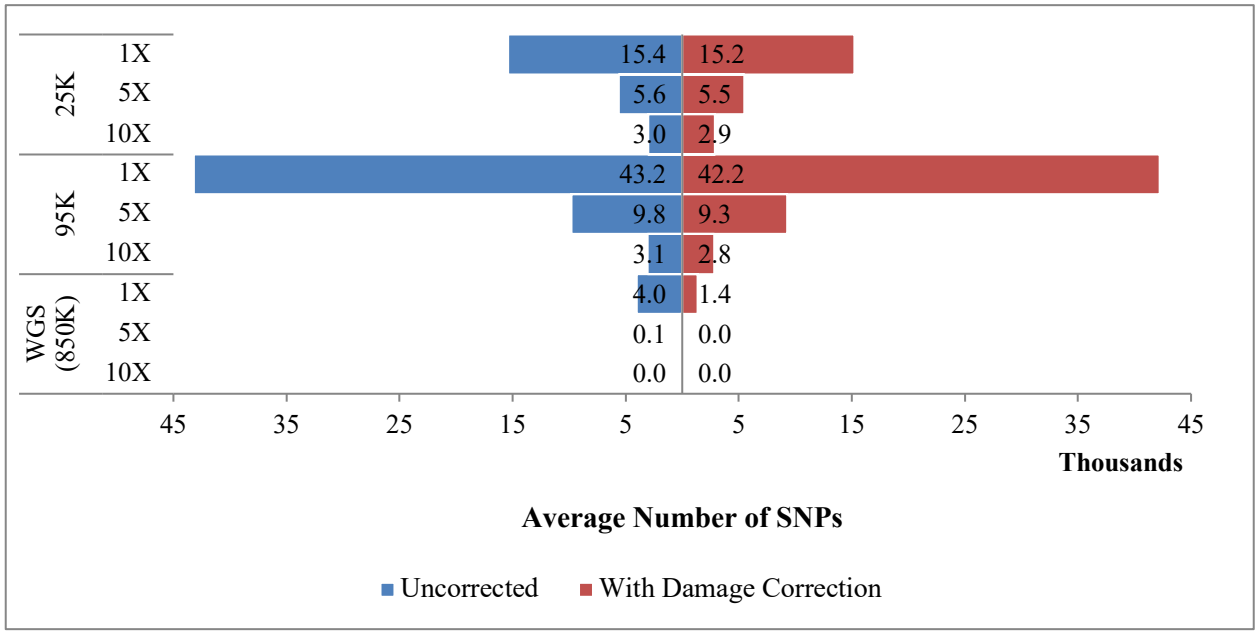

Figure 5. Average number of SNPs recovered for 14 skeletal samples, both with and without damage correction, using coverage thresholds of 1X, 5X and 10X. Only samples that were sequenced on the MiSeq are included in this comparison.

Incorporation of damage correction resulted in fewer SNPs above the specified coverage thresholds across all SNP approaches. In the merged-pair alignment, which is utilized when damage correction is applied, coverage may be reduced because merged reads with low alignment scores are removed and/or larger fragments fail to merge and thus will not be mapped. Despite this reduction, damage correction is likely to be beneficial for kinship prediction for low quality samples as the modified alignment and genotype likelihood scores take into account

450 DNA damage in order to improve the accuracy of the SNP profile. Even with the applied damage
451 correction, SNP capture successfully generated more than 10,000 SNPs for 82.1% of the non-
452 probative sample libraries (23 of 28) sequenced on the MiSeq (Table S7).
453
454 The two captured libraries sequenced on both the NextSeq and MiSeq (95K libraries for samples
455 2 and 5) yielded a similar percentage of mapped reads on both instruments, yet much smaller
456 proportions were unique in the NextSeq data (Table S7; <21% for the NextSeq and >49% for the
457 MiSeq). Although a majority of the sequencing capacity of the NextSeq is consumed by PCR
458 duplicates, the raw number of unique reads and SNP coverage per sample increased
459 substantially. Therefore, sequencing on a higher throughput instrument may be beneficial for
460 some samples with reduced SNP recovery.
461
462 *Microarray genotyping of family reference samples (FRS)*
463 DNA quantities for FRS extracts were comparable across quantification methods (Table S8),
464 averaging 1.75 ng/µl for Plexor HY and 1.70 ng/µl for Qubit HS. Overall, 35 of 39 (89.7%) FRS
465 tested were successfully genotyped, yielding SNP call rates >96%. FRS 13-A demonstrated a
466 moderate call rate (74%) but was still utilized in ancestry and kinship estimation. The three FRS
467 with call rates <70% were not included in ancestry or kinship analysis. These four reference
468 samples with call rates <96% yielded the lowest values using the Plexor HY DNA Quantification
469 Kit (<210 pg/µl), indicating human DNA quantities below the recommended input for
470 genotyping arrays. While not performed for this study, it is feasible that FRS could undergo SNP
471 capture following DNA fragmentation and library preparation in order to obtain sufficient
472 reference data. This would allow laboratories to implement a single processing method for SNP
473 genotyping (i.e. capture) for all sample types. Fχ offers capture-to-capture kinship analysis in
474 addition to the capture-to-microarray analysis used for this study.
475
476 *Parabon Fχ ancestry prediction*
477 Ancestry prediction was performed on the case samples and the FRS within the Fχ software
478 using iAdmix (37), leveraging all reads mapping to targeted SNPs. Ancestry estimates for the 36
479 FRS with sufficient call rates were determined to be European (Figure S4), consistent with the
480 self-reported race provided. The ancestry of the known references was also used as a basis for
481 the expected ancestry of the non-probative case samples. For all 25K and 95K captured samples,
482 the ancestry predicted by Fχ was European (Figures S6 and S7), consistent with the expected
483 ancestry. However, inconsistent ancestry prediction was obtained in five of 14 WGS samples
484 (Figure S5). In one WGS sample, no ancestry prediction was able to be performed due to no
485 reads being present at the 850K targeted SNPs. Inaccurate ancestry predictions, such as those
486 obtained by the WGS data in this study, could potentially be attributed to bias resulting from few
487 SNPs recovered. Since allele frequencies of the 850K targeted SNPs can vary substantially
488 between population groups, failure to account for population differences in allele frequencies can
489 impact the accuracy of kinship comparisons (38). It is important to note that this study only
490 investigated kinship between individuals of European descent. Expansion of the populations

tested, to include individuals of mixed race/ancestry, is needed in future research and validation efforts.

Parabon Fχ kinship prediction

The European allele frequency file was chosen for kinship estimation in all comparisons based on the European ancestry predictions obtained from both the FRS and the SNP capture data of the non-probative case samples. Even though the WGS data did not indicate European ancestry for all samples, the European allele frequency file was chosen since this was the expected ancestry in each instance. Since the majority of the targeted SNPs did not achieve a read depth of 5X or above, kinship comparisons were only performed using the 1X profiles. Kinship comparisons between the non-probative case samples and FRS are presented in Table 2 (21 related FRS) and Table S9 (15 unrelated FRS).

Analysis of the full 850K SNPs from the WGS data yielded few samples in which the relative and DOR were correctly predicted (Tables 2a and 2b). Only one of the 14 (7.1%) samples was predicted to be related to its true FRS at the expected DOR, while also excluding adventitious relationships to other FRS. Eight of the remaining 13 samples had adventitious hits to incorrect FRS, totaling 91 adventitious hits overall (Table 2a; Table S9). When damage correction was applied, 98.9% (90 of 91) of the adventitious hits with LRs >10,000 were removed and an additional case sample was successfully associated to its FRS. Furthermore, after damage correction the two WGS samples sequenced on the NextSeq (samples 2 and 5) enabled the correct FRS to be predicted with strong support (LR>10,000), though the DOR was inaccurate. Therefore four of 14 (28.5%) samples produced the correct *relative* after damage correction of WGS data, yet only two of 14 samples (14.2%) produced sufficient SNPs for *precise* kinship prediction indicating both the correct *relative and DOR*. Damage-corrected profiles produced false negative results in 68% of the 19 pairwise comparisons with known relatives, in that samples and their known relatives were not predicted to be related. Of the 13 false negative results, four (30%) had LRs of 1, including three 4th degree relationships and one of the 3rd degree relationships. The remaining nine of 13 false negatives had LRs between 1 and 10,000, indicating only weak support of relatedness. In seven of these nine samples with weak support for the predicted relationship (LR <10,000), the correct relative had the highest LR across all FRS. Altogether, poor quality WGS samples were shown here to yield weak or false negative results. A WGS approach to SNP genotyping may be sufficient for higher quality historical samples sequenced on a high throughput instrument such as the NextSeq. However, the majority (71.5%) of the skeletal remains tested here failed to generate sufficient data with WGS in order to predict the correct FRS with strong statistical support.

By contrast, in the 25K SNP panel uncorrected and damage-corrected data, 17 of the 21 (81.0%) FRS were correctly predicted to be related to the corresponding case sample with LRs exceeding 10,000 (Tables 2c and 2d). Of these, only two of the 17 were associated to the correct FRS with

the incorrect DOR. In other words, precise kinship estimation was possible for more than 70% of the 25K capture samples (15 of 21), regardless of DNA damage correction. The other four of 21 FRS were correctly predicted to be related to the corresponding case sample from the 25K results, but below the 10,000 LR threshold needed to demonstrate strong statistical support for relatedness. The uncorrected 95K data accurately predicted 18 relationships (85.7%), and the application of damage correction to the 95K data further increased the accuracy to 19 of 21 (90.5%) comparisons (Tables 2e and 2f). But again, two of the relationships in the 95K data had incorrectly predicted DOR. The precise kinship prediction rate from the 95K data, in which the related FRS and DOR were accurately predicted, was then 81.0% (17 of 21) of pairwise comparisons. The increased SNP recovery of the 95K panel permitted improved kinship inferences over the 25K panel, producing strong LR values for some of the pairwise comparisons in which the 25K samples yielded LRs below the 10,000 LR threshold. For example, the 95K panel resulted in a kinship association of one set of 4th degree relatives with an LR >10,000 that was not achieved with the 25K panel. Together the 25K and 95K capture data produced zero false positive relationships with LRs >10,000 in all 2,016 pairwise comparisons tested (504 per condition, excluding RBs). This includes pairwise comparisons with FRS that were unrelated to all case samples (Table S9).

As noted above, two of the kinship comparisons (sample 2 / FRS 2-B and sample 13 / FRS 13-A) predicted the correct FRS to be related but the DOR was incorrect. This result was obtained for these two comparisons regardless of the SNP capture profile conditions (25K and 95K panels as well as uncorrected and damage-corrected data). In both cases, the predicted DOR was one degree more distant than the expected relationship. The LRs obtained for these incorrect relationships were exceptionally high (averaging 8.17×10^{88} for sample 2 and FRS 2-B, and 9.99×10^{126} for sample 13 and FRS 13-A). Additionally, samples 2 and 13 both generated sufficient SNP coverage (>24,000 and >13,000 SNPs, respectively, for each of the two panels). In the case of sample 2, FRS 2-B was expected to be a grand-niece (3rd degree relative) of sample 2 but was predicted as a 4th degree relative (e.g., great-grand-niece or first cousin once removed). An incorrect classification of a 3rd degree relative is not unexpected, as the transmission of genetic material is random, and there is variation in the proportion of DNA sharing within pairs of individuals of the same DOR (38). Sample 2 obtained high LRs (averaging 1.31×10^{224}) for the correct DOR (3rd degree) with its other FRS included in the study (FRS 2-A). Moreover, the relationship predicted between FRS 2-A and FRS 2-B was 3rd degree, which is consistent with the reported relationship (first cousins) of the two FRS donors (Figure S8). Since the relationships between the sample 2 and FRS 2-A as well as the two FRS were predicted correctly, it is likely that the more distant DOR predicted for sample 2 and FRS 2-B may be the result of low allele sharing in the SNPs recovered rather than an issue with the genealogical record. By contrast, the reason for the incorrect DOR predicted for sample 13 was likely the reduced genotyping call rate of FRS 13-A (74%). In fact, the poorer quality data for FRS 13-A also led to seven adventitious hits to unrelated case samples in the WGS data (Table 2a). It is

571 therefore possible that a genotyping success threshold (e.g., >96%) should be established for
572 FRS microarray data to permit reliable kinship estimation.
573
574 *Analysis of control samples*
575 Positive controls and RBs were included in each of the sample sets to monitor the library
576 preparation and the SNP capture assays. Although the PCs were not sequenced, they were
577 assessed during several quantification steps for library product as a quality control assessment.
578 RBs were sequenced with unknown samples and analyzed to measure levels of background noise
579 as well as check for possible contamination. Kinship was compared between the RBs and the
580 FRS. Five of the six RB libraries were determined to be clean, with fewer than 100 SNPs
581 covered at the 1X threshold (Table S7), and no SNPs were recovered from any of the RBs at the
582 5X or 10X thresholds. The 25K captured product of RB1 generated 1,608 SNPs at 1X and
583 required additional investigation. Few SNPs were attributed to RB1 in the 95K and WGS data,
584 indicating a lack of human DNA present and suggesting that the library was not contaminated. It
585 is unlikely that contamination would occur during the capture step of the laboratory procedure,
586 as samples have already been indexed during library preparation. Examination of the
587 BioAnalyzer trace for RB1 showed a high adapter peak with no distinguishable capture product
588 (Figure S9), providing additional support to the conclusion that RB1 is clean. Therefore,
589 sequencing crosstalk is the most probable explanation for the reads attributed to RB1 in the 25K
590 data (17,26,27).
591
592 To further investigate the likelihood of crosstalk, a capture-to-capture kinship comparison was
593 performed between the RBs and the unknown samples (Table S10). Matches were obtained
594 between sample 2 and two RBs (RB1-25K and RB2-WGS) with LRs above 10,000 in the
595 uncorrected data; though, no match was observed once damage correction was applied. The
596 damage-corrected profile for RB1-25K matched sample 5 with a LR of 5.23×10^{35}, indicating
597 that the sequencing crosstalk likely came from this sample. Sample 5-25K and RB1-25K were
598 run together on the MiSeq instrument. Crosstalk may have been caused by the high cluster
599 density of this MiSeq run (Table S6) coupled with the sequence similarity between the indexes
600 used for RB1 and sample 5 during library preparation (Table S11). A contaminated RB from
601 suspected crosstalk could be resequenced with alternate samples to demonstrate the lack of
602 contamination in the library. However, in routine practice it may be more effective to introduce
603 an analytical threshold for RBs and samples to overcome low coverage background data
604 introduced by minimally multiplexed sequencing of captured samples and associated RBs with
605 high adapter dimers. In this particular instance of likely crosstalk, the total number of SNPs
606 covered in the RB was only 6.4% of the number of SNPs attempted (1608 out of 24,999). Thus,
607 the fractional SNP coverage was low in RB1. Only one case sample (11) dropped below this
608 fractional coverage metric with 0.64% of SNPs covered at 1X in the 25K data, and it
609 correspondingly failed to produce accurate kinship results with strong statistical support using
610 this capture assay. Therefore, it may be worthwhile to evaluate fractional SNP coverage as an

611 analytical threshold of 1X data, perhaps coverage of at least 10% of the SNPs targeted. Despite
612 the fractional coverage in RB1 for the 25K sample set, none of the RBs matched to any of the
613 FRS above the set LR>10,000 threshold under any of the conditions analyzed (Table S10).

Table 2. Likelihood ratios (LRs) were calculated for the highest predicted degree of relatedness (DOR) between each unknown and 21 family reference samples (FRS) compared to the two samples being unrelated. LRs greater than 1E+308 were reported as 1E(log LR) due to computational limitations. FRS 6-A, a relative of sample 6, failed genotyping and was excluded from kinship analysis. The remaining 15 unrelated FRS (excluding two failed FRS, 20-A and 21-A) were compared with the unknown samples and are presented in Table S9. Kinship was assessed with and without damage correction (DC) of the unknown genotypes. Analysis was performed with data generated from WGS analyzed for 850K SNP targets (3a and 3b) as well as hybridization capture for the 25K (3c and 3d) and 95K (3e and 3f) panels. Samples are organized horizontally by DOR from closest to furthest (up to 4th degree). LRs above the 10,000 threshold are bolded; true relatives to each sample are designated by a thick border. P/O = Parent/offspring; FS = Full Sibling. Sample 14-WGS yielded no reads at targeted SNPs and was not able to be compared (represented by grey boxes). Color indicates the degree of relatedness with the highest likelihood. Self = purple; P/O = yellow; FS = green; 2nd degree = orange; 3rd degree = blue; 4th degree = pink; unrelated = white

WGS-850K 1X		Sample Number														
		P/O		FS		2nd					3rd				4th	
		4	14	11	12	3	5	6	13	14	2	8	9	10	1	7
FRS #	4-A	1.40E+06		52	1	2798	2.03E+45	3	1		8.82E+137	73	1	4.10E+06	1287	1
	14-A	1		1	1	7	1	1	1		1	1	1	1	1	1
	11-A	1		1	1	1	1	1	1		1	1	1	16	1	1
	12-A	1		1	74	1	1	1	3		1	1	1	1	1	1
	3-A	2.67E+06		428	2	1891	4.33E+58	1	2951		8.26E+178	4.06E+04	1	1.17E+08	2080	6
	3-B	7.71E+05		265	5	862	3.76E+42	1	6		2.61E+148	9850	1	6.82E+06	60	1
	3-C	1.32E+06		2621	21	4151	3.41E+56	1	478		6.39E+157	245	1	2.28E+05	659	3
	3-D	2.89E+07		7301	170	1.58E+04	1.21E+51	1	424		9.52E+161	2705	1	1.61E+06	1219	4
	5-A	1.25E+05		1	24	2317	1E+432	4	9		1.80E+154	32	1	7.57E+06	181	1
	5-B	1.67E+06		35	1	537	1E+499	47	1		2.69E+156	3426	1	3.44E+05	148	1
	6-B	5.04E+06		6	1	1614	1.61E+73	1.24E+05	28		1.66E+168	34	1	4.08E+08	30	9
	13-A	2.86E+16		670	1	2.80E+05	4.49E+102	5.29E+10	1.66E+06		1E+337	1	1	2.01E+16	9.66E+06	1
	14-B	3		1	3	1	1	1	1		1	1	1	1	1	1
	2-A	1.38E+07		163	4	1079	5.64E+61	1451	7		1E+344	3	1	7.07E+06	5.97E+05	1
	2-B	6.33E+05		142	14	127	2.27E+40	9	2		4.44E+288	1016	1	1.83E+06	6604	1
	8-A	1		1	17	1	1	1	1		1	1	2	1	1	1
	9-A	1		1	1	1	1	1	1		1	1	1.30E+14	1	1	1
	10-A	1		1	4	1	1	1	1		1	1	1	2	1	1
	1-A	1.60E+06		20	20	120	1.43E+47	11	7		1.03E+146	19	1	1.26E+05	9182	1
	1-B	4.89E+05		37	32	962	1.02E+52	25	15		2.19E+150	41	1	5.07E+06	1178	1
	7-A	1		1	1	13	1	1	1		1	1	1	1	1	1

WGS-850K 1X-DC		Sample Number														
		P/O		FS		2nd					3rd				4th	
		4	14	11	12	3	5	6	13	14	2	8	9	10	1	7
FRS #	4-A	2		1	1	1	1	14	1		1	3	1	2	1	1
	14-A	1		1	1	5	1	1	1		1	1	1	1	1	1
	11-A	1		3	1	1	1	1	1		1	1	1	1	1	1
	12-A	1		1	2.24E+10	1	1	1	2		1	2	1	1	2	1
	3-A	1		1	1	21	1	2	2		1	8	1	8	1	1
	3-B	1		1	1	52	1	1	1		1	8	1	2	1	1
	3-C	1		1	1	17	1	8	1		1	4	1	1	1	1
	3-D	1		1	5	691	1	4	1		1	2	1	4	1	1
	5-A	1		1	3	1	1E+424	3	1		1	2	1	4	2	1
	5-B	1		1	1	3	1E+495	2	1		1	5	1	2	1	1
	6-B	1		1	1	2	4.27E+05	72	2		1	2	1	12	1	1
	13-A	1		1	1	1	1	2	27		1	1	1	4	11	1
	14-B	1		1	1	1	1	1	1		1	1	2	2	1	2
	2-A	1		2	3	2	1	1	1		1.18E+170	2	1	23	1	1
	2-B	1		1	1	2	1	13	1		9.00E+114	4	1	1	1	1
	8-A	1		4	2	1	1	1	1		1	1	9	2	1	1
	9-A	1		1	1	1	1	1	1		1	1	1.74E+15	1	1	1
	10-A	38		1	6	1	1	1	1		1	1	1	33	1	1
	1-A	1		1	1	1	1	2	1		1	2	1	7	1	1
	1-B	1		1	2	1	1	3	1		1	4	1	4	1	1
	7-A	1		1	1	1	1	1	1		1	1	1	1	1	1

25K 1X	Sample Number														
	P/O		FS		2nd					3rd				4th	
FRS #	4	14	11	12	3	5	6	13	14	2	8	9	10	1	7
4-A	5.75E+259	1	4	1	1	1	1	1	1	1	1	1	1	17	1
14-A	1	1E+466	1	1	1	1	1	87	1E+466	1	1	1	1	1	1
11-A	1	1	76	1	1	1	1	1	1	1	1	1	1	1	1
12-A	1	1	2	1E+734	1	1	1	1	1	1	1	1	1	1	1
3-A	1	1	1	1	6.73E+266	1	1	1	1	1	1	1	1	1	1
3-B	1	1	5	1	6.47E+253	1	1	1	1	1	1	1	1	1	1
3-C	1	1	1	1	1.14E+290	1	1	1	1	1	1	1	1	1	56
3-D	1	1	1	1	3.68E+299	1	1	1	1	1	1	1	1	1	1
5-A	1	1	1	1	1	9.26E+304	1	2	1	1	1	1	1	11	1
5-B	1	1	1	1	1	1E+420	1	1	1	1	1	1	1	1	1
6-B	1	1	1	1	1	1	8.83E+179	1	1	1	1	1	1	1	1
13-A	1	1	1	1	1	1	1	4.16E+42	1	1	1	1	4	1	1
14-B	1	2.70E+113	1	1	1	1	1	1	2.70E+113	1	1	1	1	1	2067
2-A	1	1	2	1	1	1	1	1	1	2.73E+101	1	1	1	1	1
2-B	1	1	1	1	1	1	1	1	1	1.68E+36	1	1	1	1	8
8-A	1	1	1	1	1	1	1	1	1	1	2.63E+23	1	1	1	1
9-A	1	1	1	1	1	1	8	1	1	1	1	1.48E+99	1	1	1
10-A	1	1	1	1	1	1	1	1	1	1	1	1	2.51E+68	1	1
1-A	1	1	1	1	1	1	1	1	1	1	1	1	1	202	1
1-B	1	1	1	1	1	1	1	1	1	1	1	1	1	165	1
7-A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	4665

27

25K 1X-DC	Sample Number														
	P/O		FS		2nd					3rd				4th	
FRS #	4	14	11	12	3	5	6	13	14	2	8	9	10	1	7
4-A	4.55E+264	1	2	1	1	1	1	1	1	1	1	1	1	6	2
14-A	1	1E+464	1	1	1	1	1	87	1E+464	1	1	1	1	1	1
11-A	1	1	985	1	1	1	1	1	1	1	1	1	1	1	1
12-A	1	1	1	1E+726	1	1	1	1	1	1	1	1	1	1	1
3-A	1	1	1	1	1.66E+260	1	1	1	1	1	1	1	1	1	1
3-B	1	1	1	1	3.65E+248	1	1	1	1	1	1	1	1	1	1
3-C	1	1	1	1	3.07E+280	1	1	1	1	1	1	1	1	1	39
3-D	1	1	1	1	8.14E+287	1	1	1	1	1	1	1	1	1	1
5-A	1	1	1	1	1	1E+313	1	2	1	1	1	1	1	32	1
5-B	1	1	1	1	1	1E+428	1	1	1	1	1	1	1	1	1
6-B	1	1	1	1	1	1	7.64E+180	1	1	1	1	1	1	1	1
13-A	1	1	6	1	1	1	1	5.37E+42	1	1	1	1	1	1	1
14-B	1	1.70E+114	1	1	1	1	1	1	1.70E+114	1	1	1	1	1	2266
2-A	1	1	2	1	1	1	1	1	1	1.09E+101	1	1	1	4	1
2-B	1	1	1	1	1	1	1	1	1	1.44E+36	1	1	1	1	3
8-A	1	1	1	1	1	1	1	1	1	1	1.06E+23	1	1	1	1
9-A	1	1	1	1	1	1	10	1	1	1	1	6.08E+97	1	1	1
10-A	1	1	1	1	1	1	1	1	1	1	1	1	1.73E+65	1	1
1-A	1	1	1	1	1	1	1	1	1	1	1	1	1	255	1
1-B	1	1	1	1	1	1	1	1	1	1	1	1	1	694	1
7-A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3532

28

95K 1X		Sample Number														
		P/O		FS		2nd					3rd				4th	
		4	14	11	12	3	5	6	13	14	2	8	9	10	1	7
FRS #	4-A	2.05E+208	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	14-A	1	1E+1053	1	1	1	1	1	1	1E+1053	1	1	1	1	1	1
	11-A	1	1	13	1	1	1	1	1	1	1	1	1	1	1	1
	12-A	5	1	1	1E+1740	1	1	1	1	1	1	1	1	1	1	1
	3-A	1	1	1	1	1E+605	1	1	1	1	1	1	1	1	1	1
	3-B	1	1	1	1	1E+631	1	1	1	1	1	1	1	1	2	1
	3-C	1	1	1	1	1E+612	1	1	1	1	1	1	1	1	1	1
	3-D	1	1	1	1	1E+719	1	1	1	1	1	1	1	1	1	1
	5-A	2	1	1	1	1	1E+432	1	1	1	1	1	1	1	1	1
	5-B	1	1	1	1	1	1E+529	1	1	1	1	1	1	1	1	1
	6-B	1	1	1	1	1	1	4.47E+242	1	1	1	1	1	1	1	1
	13-A	9562	1	1	1	1	1	1	4.00E+127	1	1	1	1	1	1	55
	14-B	1	2.91E+303	2	1	1	1	1	1	2.91E+303	1	1	1	1	1	1
	2-A	1	1	1	1	1	1	1	1	1	4.12E+224	1	1	1	1	1
	2-B	1	1	1	1	1	1	1	1	1	3.24E+89	1	1	1	1	2
	8-A	1	1	1	1	1	1	1	1	1	1	3.84E+62	1	1	1	35
	9-A	1	1	1	1	1	1	1	1	1	1	1	1.85E+250	1	1	1
	10-A	1	1	1	1	1	1	1	1	1	1	1	1	5.27E+170	1	1
	1-A	1	1	6	1	1	1	1	1	1	1	1	1	1	167	1
	1-B	1	1	1	1	1	1	1	1	1	1	1	1	1	1.05E+04	1
	7-A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	259

29

95K 1X-DC		Sample Number														
		P/O		FS		2nd					3rd				4th	
		4	14	11	12	3	5	6	13	14	2	8	9	10	1	7
FRS #	4-A	1.87E+219	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	14-A	1	1E+1077	1	1	1	1	1	1	1E+1077	1	1	1	1	1	1
	11-A	1	1	1.04E+11	1	1	1	1	1	1	1	1	1	1	1	1
	12-A	4	1	1	1E+1723	1	1	1	1	1	1	1	1	1	1	1
	3-A	1	1	1	1	1E+594	1	1	1	1	1	1	1	1	1	2
	3-B	1	1	1	1	1E+617	1	1	1	1	1	1	1	1	3	1
	3-C	1	1	1	1	1E+598	1	1	1	1	1	1	1	1	1	1
	3-D	1	1	1	1	1E+700	1	1	1	1	1	1	1	1	1	1
	5-A	5	1	1	1	1	1E+434	1	1	1	1	1	1	1	1	1
	5-B	1	1	2	1	1	1E+520	1	1	1	1	1	1	1	1	1
	6-B	1	1	1	1	1	1	9.47E+241	1	1	1	1	1	1	1	1
	13-A	32	1	1	1	1	1	1	1.08E+116	1	1	1	1	1	1	11
	14-B	1	4.02E+305	1	1	1	1	1	1	4.02E+305	1	1	1	1	1	1
	2-A	1	1	1	1	1	1	1	1	1	1.12E+224	1	1	1	1	1
	2-B	1	1	1	1	1	1	1	1	1	2.59E+87	1	1	1	1	2
	8-A	1	1	3	1	1	1	1	1	1	1	1.17E+64	1	1	1	30
	9-A	1	1	1	1	1	1	1	1	1	1	1	4.05E+251	1	1	1
	10-A	1	1	1	1	1	1	1	1	1	1	1	1	2.77E+162	1	1
	1-A	1	1	3	1	1	1	1	1	1	1	1	1	1	208	1
	1-B	1	1	1	1	1	1	1	1	1	1	1	1	1	1.01E+04	1
	7-A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2340

30

631 **Discussion**
632 This study demonstrates that adequate SNP data can reliably be obtained from aged skeletal
633 samples by employing hybridization capture followed by NGS. The SNP recovery in
634 combination with the Parabon Fχ Forensic Analysis Platform were sufficient to permit accurate
635 distant kinship predictions between severely degraded bone samples and known relatives.
636 Though microarray testing will be successful for many forensic-type samples, DNA degradation
637 combined with low endogenous DNA content render aged skeletal remains recalcitrant to this
638 traditional genotyping method. Also, as demonstrated in this study, reference samples may fail to
639 generate reliable microarray data and thus require alternative genotyping methods due to lower
640 DNA quality/quantity. Hybridization capture can be combined with a fragmenting library
641 preparation method for high-quality samples, which would allow a single SNP genotyping
642 method to be implemented for all sample types – lessening the validation burden for the
643 laboratory.
644
645 In general, the poor SNP recovery generated from WGS of the library molecules demonstrates
646 the low quantity of human DNA present in historical bone samples, and this reinforces that target
647 enrichment is needed for abundant SNP acquisition. Results from the capture data illustrate that,
648 for most samples, the percentage of reads covering targeted SNPs is remarkably similar between
649 the 25K and 95K panels, despite the difference in the number of probes targeting each SNP (four
650 versus two baits, respectively). SNP recovery is thus proportional to the number of SNPs
651 targeted in the panel. As it is impossible to determine which SNPs may be recovered from
652 sample libraries, it benefits the kinship analysis to probe for the maximum number of SNPs
653 possible. The Parabon Fχ software facilitates this maximal SNP capture approach by
654 implementing a probabilistic SNP genotyping algorithm for low coverage sequence data that is
655 tailored for degraded DNA samples with cytosine deamination. The maximum SNP probe
656 capacity may be determined by factors such as the number of probes that can be effectively put
657 in the capture assay, which must be balanced with cost and sequencing throughput. The present
658 study demonstrates only a moderate (10%) increase in the proportion of precisely predicted
659 relationships when increasing from the 25K to the 95K panel, which is roughly 50% more
660 expensive. Although the cost of a custom capture panel is related to the bait design and number
661 of reactions purchased, in the present study an order for 96 capture reactions would cost $17,760
662 for the 25K panel (four baits per SNP) versus $26,400 for the 95K panel (two baits per SNP).
663 Laboratories pursuing hybridization capture should weigh the benefits of maximum SNP
664 recovery with other factors including expected DOR, sample type, sequencing throughput, and
665 budget.
666
667 A 25K SNP panel generated suitable data to accurately associate up to 3rd degree relatives with
668 the correct DOR prediction and strong statistical support. The recovery of additional SNPs with
669 the 95K SNP panel permitted accurate association of up to 4th degree relatives, the furthest DOR
670 tested. WGS was less successful in obtaining the requisite coverage needed for kinship

comparison when sequencing was performed on a MiSeq instrument, as a majority of samples produced false negative results after damage correction was applied. Furthermore, the WGS data, even with damage correction, produced one false positive result / adventitious hit with a LR >10,000 indicating strong statistical support for an incorrect relationship between a case sample and an unrelated FRS. Adventitious hits are expected and likely cannot be avoided, especially in large-scale databases (2) and when dense marker sets are used (39). In the capture data (both 25K and 95K panels), no adventitious hits were generated when using an LR threshold > 10,000. However, false negative results were produced in four of 21 pairwise comparisons due to low LR values (<5,000). It is of larger consequence to misclassify two individuals as related than the alternative. Therefore it is imperative to choose laboratory methods and appropriate interpretation thresholds (e.g., LR, proportion of SNPs recovered) that minimize the number of false positives obtained.

In practice, it may be beneficial for the LR threshold to be based on simulations that determine the maximum expected value from unrelated individuals for a particular DOR. Additionally, the utility of calculating the posterior probability to provide statistical weight to the conclusions should be considered. Incorporation of the posterior probability into the interpretation of the results may be particularly useful in instances where the kinship analysis distinctly indicates relatedness, yet the predicted DOR is inconsistent with reported familial relationships. There are many explanations for noted differences between expected relationships and results obtained through genetic kinship analyses. The distribution of shared DNA varies widely among individuals of the same DOR, and the degree of overlap between different DORs increases as the relationships become more distant (40). Alternatively, there may be issues with genealogical records, such as reporting flaws, non-paternity, or incorrect pedigrees. Policies should be in place for addressing these scenarios in light of sensitive family situations, while retaining the ability to identify the unknown individual (e.g., testing additional references) (2).

The SNP genotypes obtained from historical samples exhibit cytosine deamination and are similar in quality to aDNA. For this reason, the damage correction option is a critical feature of the Parabon Fχ software that enhances the accuracy of the SNP genotypes. When damage correction was applied to the data herein, the predicted DOR was improved. Previous reports have demonstrated through simulation that genotyping errors have a substantial effect on the LR of kinship scenarios, particularly for close genetic relationships (39). The DNA damage expected to be observed in historical remains and aged skeletal samples can affect read alignment to the reference genome, especially in short fragments, as well as SNP genotyping, thereby culminating in genotyping errors. Erroneous SNP alleles could increase allele sharing and artificially inflate kinship statistics (39), leading to adventitious hits between unrelated individuals such as those observed in the WGS data before damage correction. When both SNP capture and damage correction were used in combination on the historical samples in this study, in no instance was a casework sample incorrectly paired with an unrelated FRS (LR >10,000).

This approach of combining large-scale SNP capture with the Parabon Fχ software tailored for degraded DNA analysis provided promising results for forensic genetics in this study, particularly for historical remains cases. SNP capture from aged, degraded skeletal samples may be especially impactful in cases where DNA degradation has prevented successful STR amplification and/or where there is a lack of paternal or maternal relatives. The ability to capture requisite SNP data overcomes STR limitations, expands the pool of eligible DNA sample donors suitable for kinship comparisons by enabling distant kinship estimation from living relatives and removes the need to use lineage assays (mitochondrial DNA sequencing or Y-chromosomal STRs). Although further testing and validation are still required before implementation in a forensic laboratory, this study demonstrates the successful application of SNP capture combined with the Parabon Fχ software to facilitate distant kinship estimation in decades-old unidentified remains cases.

Disclaimer

The assertions herein are those of the authors and do not necessarily represent the official position of the United States Department of Defense, the Defense Health Agency, or its entities including the Armed Forces Medical Examiner System. Any mention of commercial products was done for scientific transparency and should not be viewed as an endorsement of the product or manufacturer

Conflicts of interest

Steven Armentrout, Ellen McRae Greytak, and Janet Cady are employees of Parabon NanoLabs, Inc., the developer and commercial vendor of the Parabon Fχ Forensic Analysis Platform.

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Data availability

Data are on file at the AFMES-AFDIL and available to authorized third-party scientists upon written request.

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760
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