Received: 28 February 2022 | Revised: 29 March 2023 | Accepted: 8 June 2023

DOI: 10.1002/elps.202200052

ELECTROPHORESIS

RESEARCH ARTICLE

Mitochondrial DNA control region typing from highly degraded skeletal remains by single-multiplex next-generation sequencing

Diana C. Vinueza-Espinosa | **Daniel R. Cuesta-Aguirre** | **Assumpció Malgosa** | **Cristina Santos**

Research Group in Biological Anthropology, Biological Anthropology Unit, Department of Animal Biology, Vegetal Biology and Ecology, Universitat Autònoma de Barcelona, Bellaterra, Spain

Correspondence
Cristina Santos, Research Group in Biological Anthropology, Unitat d'Antropologia Biològica, Department de Biologia Animal, Biologia Vegetal i Ecologia, Facultat Biociències. Edifici C, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Barcelona, Spain.
Email: cristina.santos@uab.cat

Color online: See the article online to view Figure 2 in color.

Funding information
Ministerio de Ciencia e Innovación, Grant/Award Number: PGC2018-096666-B-100; Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR), Grant/Award Number: 2021 FI_B1 00064; Generalitat de Catalunya, Grant/Award Numbers: 2017 SGR 1630, 2021 SGR 00186

Abstract

Poor nuclear DNA preservation from highly degraded skeletal remains is the most limiting factor for the genetic identification of individuals. Mitochondrial DNA (mtDNA) typing, and especially of the control region (CR), using next-generation sequencing (NGS), enables retrieval of valuable genetic information in forensic contexts where highly degraded human skeletal remains are the only source of genetic material. Currently, NGS commercial kits can type all mtDNA-CR in fewer steps than the conventional Sanger technique. The PowerSeq CRM Nested System kit (Promega Corporation) employs a nested multiplex-polymerase chain reaction (PCR) strategy to amplify and index all mtDNA-CR in a single reaction. Our study analyzes the success of mtDNA-CR typing of highly degraded human skeletons using the PowerSeq CRM Nested System kit. We used samples from 41 individuals from different time periods to test three protocols (M1, M2, and M3) based on modifications of PCR conditions. To analyze the detected variants, two bioinformatic procedures were compared: an in-house pipeline and the GeneMarker HTS software. The results showed that many samples were not analyzed when the standard protocol (M1) was used. In contrast, the M3 protocol, which includes 35 PCR cycles and longer denaturation and extension steps, successfully recovered the mtDNA-CR from highly degraded skeletal samples. Mixed base profiles and the percentage of damaged reads were both indicators of possible contamination and can provide better results if used together. Furthermore, our freely available in-house pipeline can provide variants concordant with the forensic software.

Abbreviations: CR, control region; mtDNA-CR, mitochondrial DNA control region; NGS, next-generation sequencing; PMDS, PMD score.

KEYWORDS
mtDNA, NGS, PowerSeq CRM Nested System kit, protocol optimization, skeletal samples

1 | INTRODUCTION

Genetic identification of highly degraded human skeletal remains is challenging due to the small amount of DNA present, and due to fragmentation, which ranges from 35 base pairs (bp) to 150 b [1]. This often hinders the complete recovery of autosomal short tandem repeats, which are highly informative for individual identification. However, in cases of identification involving kinship matching, other genome regions could be used, such as mitochondrial DNA (mtDNA). The higher copy number of mtDNA per cell (100–1000 copies) compared to two copies per cell of nuclear DNA increases the retrieval of genetic information to support the identification process, which can associate remains with maternal lineages [2, 3].

The mitochondrial DNA control region (mtDNA-CR) has been analyzed for a long time for forensic purposes using Sanger sequencing. The mtDNA-CR typically consists of about 1122 bp with high levels of variation, especially in the two hypervariable segments, HVS-I and HVS-II. When sequencing high-quality samples using Sanger technology, two distinct regions of around 200–400 bp are usually amplified by polymerase chain reaction (PCR). However, attempting to obtain >300 bp amplicons from poor-quality specimens may not yield any results as the DNA is highly fragmented into smaller molecules [4, 5]. Recovery of small amplicons (100–130 bp) has been successfully demonstrated in ancient DNA research and forensic analysis of bones and hair shafts [6]. Some authors have demonstrated that the use of a set of shorter amplicons is an effective alternative for the analysis of highly degraded samples. However, at least eight fragments must be amplified and sequenced to recover the entire CR, representing at least 8 PCR amplification reactions and 16 Sanger sequencing reactions [4, 7]. This makes the procedure very demanding and time-consuming. In recent years, next-generation sequencing (NGS) or massively parallel sequencing has emerged as highly robust techniques for typing mtDNA sequences in forensics and ancient DNA [8–13].

NGS provides a more extensive and informative genetic dataset with higher throughput by sequencing millions of small fragments of DNA in parallel. The fragments can be sequenced several times, providing high accuracy and insight into sequencing errors, mixture interpretation, and molecular damage. This technology also presents a lower cost per nucleotide than capillary electrophoresis-based methods [14].

Bioinformatic analyses are used to piece together the generated NGS fragments by mapping the individual reads to the human reference genome, resulting in several reads per position. Moreover, advances in bioinformatic analysis applied to ancient DNA now offer new methods to detect contamination and authenticate the results [15], although these are not currently being used in forensic routines.

NGS has yet to be widely adopted in forensic laboratories. Several commercial NGS kits are available for mtDNA-CR analysis and have been used successfully in highly degraded skeletal remains [12]. The ForenSeq mtDNA-CR Kit (Verogen) [16] can recover 18 overlapping amplicons of 60–150 bp using 18 primer pairs distributed in 2 multiplexes. The Precision ID mtDNA-CR Panel Kit (Thermo Fisher) [17] can recover 14 amplicons of 163 bp using 14 primer pairs distributed in 2 multiplexes. In both kits, two PCR reactions are performed to amplify the whole mtDNA-CR, followed by adapter ligation and sample indexing.

The PowerSeq CRM Nested System kit (Promega Corporation) has the advantage of simplifying workflow using a nested amplification protocol. It does so by employing a multiplex-PCR strategy to amplify the whole mtDNA-CR in a single multiplex that combines 10 primer pairs, generating 10 overlapping short amplicons (147–237 bp), and incorporates indexed adapters for Illumina MiSeq (Illumina, Inc., San Diego, CA, USA) in only one PCR reaction step. This process essentially reduces amplicon manipulation and risk of contamination. The Federal Bureau of Investigation (FBI) Laboratory has validated the technique according to the SWGDAM (Scientific Working Group on DNA Analysis Methods) guidelines for forensic DNA [18]. The importance of the FBI's validation is also that the kit achieved NDIS approval, which is a big deal for US labs as it can be used for mtDNA data generation and upload to the CODIS database. Moreover, some studies have demonstrated the robustness of this kit in cases with low quantities and highly degraded mtDNA. Successful coverage of 99.29% of the mtDNA-CR could be obtained at 50× coverage or more from human remains dated from the 9th to 18th centuries [3]. Another study, which aimed to identify damage patterns in the mtDNA-CR using this kit, demonstrated that low template samples are more susceptible to damage, leading to the identification of damage sites caused by molecular damage or heteroplasmy [19].

Working with fragmented DNA is just one of the challenges when dealing with older human skeletal material. Other concerns related to the authenticity of results, including the confirmation of the presence of endogenous DNA molecules, the reduction of contamination, and distinguishing among molecular damage, contamination, or mixed profiles, are particularly crucial for remains recovered from mass graves [20]. Some authenticity criteria for DNA from ancient and forensic samples were established several years ago [21, 22]. However, the ancient DNA community has incorporated bioinformatic tools to authenticate endogenous DNA, mainly based on patterns of DNA damage across genomic sequences generated by NGS [23]. These tools can eliminate PCR duplicates (essentially in PCR based techniques) and can identify the DNA damage markers that are typically observed in ancient samples [11, 15, 24–30].

We have been working with the PowerSeq CRM Nested System kit (Promega Corporation), and the standard protocol has yielded poor results in ancient samples [31].

Hence, in this study, we aim (i) to analyze the success of mtDNA-CR typing of highly degraded human skeletal remains, from different periods from the Neolithic until the 20th century, using the PowerSeq CRM Nested System kit, testing controlled library protocol changes; (ii) to implement authenticity criteria, as usually used in ancient DNA, in bioinformatic data analysis processes (in-house pipeline) that will enable an evaluation of the authenticity of results; and (iii) to compare the variant call obtained with the in-house pipeline using GeneMarker HTS software [32] (most recently released software package used by the forensic community).

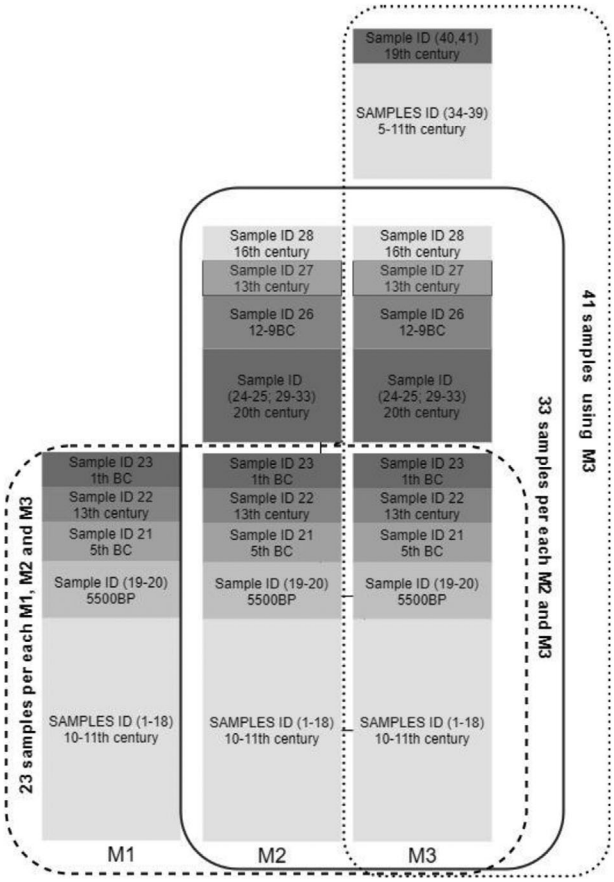

FIGURE 1 Details of the samples from different periods used to evaluate the recovery of mitochondrial DNA control region (mtDNA-CR) typing in each next-generation sequencing (NGS)-protocol M1, M2, and M3.

2 | MATERIALS AND METHODS

2.1 | Sample preparation and DNA extraction

Samples of bones and teeth were selected from 41 individuals from different archaeological contexts from 5500 BP–16th century (ancient samples) until the 19–20th century (contemporary samples) (Figure 1).

The skeletal remains (bone and teeth) were cleaned by removing the external surface with a sterile tungsten tip (previously cleaned with soap, alcohol, distilled water, and bleach and sterilized by autoclave and irradiated with UV) placed in a micro drill up to 5000 rpm. Then roughly 150 mg of the sample was cut using a sterile tungsten disk and fragmented with a forceps to obtain smaller 10 mm pieces. These were subsequently pulverized to powder by mortar to make sample digestion more accessible in the DNA extraction procedure. DNA was extracted using a silica-based method on an HE-membrane column [33] in the Ancient DNA Laboratory at the *Universitat Autònoma de Barcelona* (UAB).

2.2 | Amplification and library preparation using PowerSeq CRM kit (10-plex) and sequencing

In the first experimental phase (Figure 1), 23 samples were used to test 3 NGS-protocols based on modifications of PCR conditions: (i) The **M1** protocol was performed following the manufacturer's recommendation: 96°C for 10 min, 30 cycles of 96°C for 5 s, 60°C for 35 s, 72°C for 5 s, and 60°C for 2 min; (ii) the **M2** protocol was based on the same conditions as the M1 protocol, but increasing the number of cycles to 35; (iii) the **M3** protocol included 35 cycles and longer denaturation and extension steps: 96°C for **15 min**,

35 cycles of 96°C for **15 s**, 60°C for 35 s, 72°C for **30 s**, and 60°C for 2 min.

Ten additional samples were tested using the M2 and M3 protocols in the second phase. In total, 33 samples were tested using the M2 and M3 protocols. Eight additional samples were amplified using only the M3 protocol in the final phase. Therefore, 41 samples were tested using the M3 protocol (Figure 1).

Amplification reactions-libraries were purified using the Qiagen GeneRead size selection kit (column-based protocol) following the manufacturer's protocol (*GeneRead Size Selection of DNA Libraries Prepared*). Library quantifications were performed with the Qubit dsDNA BR Assay Kit. The Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies) was used to obtain the distributions of NGS-libraries by the length of amplicons recovered. The samples that presented a library concentration <0.005 ng/μL by Qubit were not sequenced by NGS. The libraries that were sequenced were normalized to 1 nM. Three sequencing runs were performed in the Illumina MiSeq instrument using standard flow cells MiSeq Reagent Kit v2 Nano 2 × 150 bp.

2.3 | NGS-data analysis

2.3.1 | Alignment and variant call

FASTQ *PowerSeq CRM* sequencing data were analyzed using GeneMarker HTS version 2.5.0 software and an in-house pipeline developed by our group that includes a removal of duplicates and postmortem molecular damage analyses (available online at https://github.com/DanielRCA/MTDNA-CR).

The in-house pipeline consisted of quality-checked FASTQ by FastQC v0.11.9 [34]. Duplicates were eliminated with a fastp v0.23.2 analysis. Adapters and primers were eliminated with another two consecutive fastp analyses. Subsequently, overlapping reads were merged with a final fastp analysis. Reads were aligned to the revised Cambridge Reference Sequence (GenBank accession NC_012920.1) using BWA v0.7.17 [35]. The sequence was linearized from bases 15 901 to 700 to avoid problems caused by the circularity of the mtDNA and by the reads aligned in the replication origin. SAMtools v1.16.1 was used to generate mapped, sorted, and quality-checked $Q > 30$ BAM files. BAM-quality was checked by QualiMap v.2.2.2.a, after which the variants were called using freebayes v1.3.6 and vcflib v1.0.3.

The GeneMarker HTS version 2.5.0 software package was used to analyze FASTQ sequences considering a minimum base call quality score of $Q < 30$ (bases that scored less than 30 were trimmed from 3′ after the reads).

Variant call was performed considering a minimum depth coverage of 10 and a minor variant frequency of 30%, meaning that only positions with a minimum of 10 reads were considered and that a mixed base is called if 30% of the total reads of the position represent a minor variant. In the case of the in-house pipeline, duplicated reads were previously excluded, whereas with GeneMarker HTS version 2.5.0, all the reads were considered. Indels, multi-nucleotide polymorphisms, and complex events (bases between positions 303 and 315) were not considered. The variants or haplotypes were visualized and verified using the integrative genomics viewer (IGV) v.2.9.4 [36]. HaploGrep 2 v2.4.0 was used to predict the mtDNA haplogroup based on mtDNA-CR variant call [37].

2.3.2 | Postmortem molecular damage

NGS data generated with the PowerSeq CRM Nested System kit for bone/tooth from 18 well-preserved individuals from the 20th century and buccal swabs from 12 present-day individuals were used to establish reference values for postmortem molecular damage, using the statistical framework previously described by Skoglund et al. [24]. In brief, to investigate the authenticity of a given DNA fragment, Skoglund et al. [24] evaluated two competing models, one of which assumes ancient DNA degradation, whereas the other does not, arriving at a final log-likelihood ratio of the two models that they call the PMD score (PMDS). A positive PMDS for a DNA fragment indicates support for the ancient DNA model relative to the alternative model. In this context, PMDtools v0.60 [24] was used, considering a PMDS of 1, to obtain reference values for damage in the present day and in samples from the 20th century. Moreover, PMDtools v0.60 was used to extract reads with molecular damage for all the samples analyzed with different methods. Values obtained were compared with the established limits for the present day and 20th century in order to test the recovery of degraded DNA and data authentication.

2.4 | Evaluation of parameters

The following parameters were evaluated to compare library methods, evaluate data authenticity, compare amplicon performance, and compare variant call between bioinformatic tools:

–Percentage of valid libraries: percentage of libraries with a concentration higher than 0.005 ng/μL.

–Mean depth coverage excluding duplicated reads for all positions of mtDNA-CR: mean of reads by position, considering all positions of mtDNA-CR.
–Percentage of mtDNA-CR recovered: positions recovered in relation to the total number of positions of mtDNA-CR. The recovered positions were calculated using two criteria: positions with a minimum depth coverage higher than 30 reads (30×) and positions with a minimum depth coverage higher than 10 reads (10×).
–Percentage of genetic information recovered by the type of amplicon: similar to the percentage of mtDNA-CR recovered but considering each amplicon separately.
–Percentage of damaged reads: number of damaged reads divided by mapped reads without duplicates.
–Number of mixed bases: obtained from variant call with the two bioinformatic tools, considering positions with a minimum depth coverage of 10 and in which a mixed base is reported if the minor variant represents 30% of the reads of a position.
–Concordance of the variants called using the in-house pipeline and GeneMarker HTS: Variant call was performed considering positions with a minimum depth coverage of 10 and in which a mixed base is reported if the minor variant represents 30% of the reads. In the case of the in-house pipeline, duplicated reads were not considered.

3 | RESULTS

3.1 | Comparison M1/M2/M3 and M2/M3

Table S1 presents results for samples included in phases 1 and 2. Parameters of library quantification, sequence quality and recovery recovered, and molecular damage, obtained with the in-house pipeline, were presented.

3.1.1 | Percentage of valid libraries

Table 1 reports the number and percentage of libraries with a concentration higher than 0.005 ng/μL that were considered valid and were further sequenced in experimental phases 1 and 2. From the 23 samples analyzed using M1, M2, and M3 methods (phase 1), only 6 libraries (26%) obtained with M1 were valid. In contrast, 22 libraries (95.7%) and 23 libraries (100%), respectively, for M2 and M3, were considered valid for sequencing. Focusing only on M2 and M3 (phase 2), the 33 samples analyzed using both methods again revealed better results for M3 (Table 1).

TABLE 1 Number and percentage of valid libraries obtained in experimental phases 1 and 2, according to the different methods used in library preparation (M1, M2, and M3).

Experimental phase	Number of samples	Valid libraries *N* (%)		
		M1	M2	M3
Phase 1: M1/M2/M3	23	6 (26)	22 (95.7)	23 (100)
Phase 2: M2/M3	33	–	31 (93.9)	33 (100)

Note: M1 protocol: manufacturer's recommendation. M2 protocol: manufacturer's recommendation with 35 cycles. M3 protocol: 35 cycle and longer denaturation and extension steps.

The retrieval profile of the libraries by the length of amplicons is shown in Figure 2. Amplicons between 160 and 230 bp were obtained using the M2 and M3 protocol, whereas M1 showed amplicons of 68 bp that correspond to primer-dimers, and just one peak of 220 bp. Although both M2 and M3 protocols showed amplicons recovered with the required size for NGS-sequencing, M3 produces a greater quantity of each amplicon than M2 (Figure 2).

3.1.2 | Mean depth coverage including all positions of mtDNA-CR and percentage of mtDNA-CR recovered

Table 2 reports the mean depth coverage and the percentage of mtDNA-CR recovered for samples used in phase 1 and that have sequence results for the three protocols tested. All the indicators considered data without duplicates. The high heterogeneity between samples is evidenced, but a consistent increase in the amount of sequence generated with the change to standard protocol M1 is observed.

Table S1 reports the values of the mean depth coverage and the percentage of mtDNA-CR recovered for all the samples and libraries analyzed in phases 1 and 2. The comparison of results obtained for samples used in phase 2 (analyzed using M1 and M2) evidence a consistent increase from M2 to M3 in mean depth coverage (increased mean value of 226 reads). As for the percentage of mtDNA-CR recovered considering only positions with a depth coverage of 30 (30×) or 10 (10×), an increase of around 20% in the extension of the region recovered is observed from M2 to M3.

The recovered region represents less than 15% of the total mtDNA-CR (less than 200 bp) in many samples analyzed with M2 in comparison to M3, particularly when a by posi-

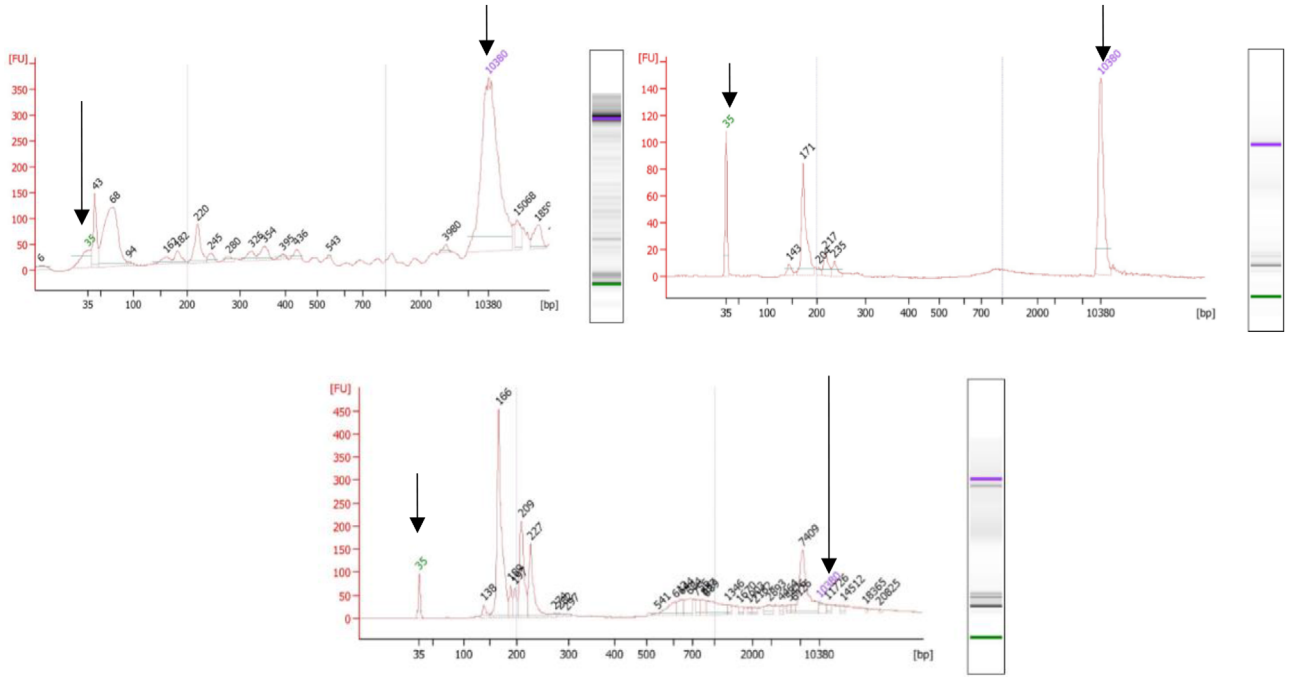

FIGURE 2 Length fragment distribution of libraries performed for Sample ID 4 using the three next-generation sequencing (NGS)-protocols. Size markers or ladders are indicated by arrows at 35 and 10 380 bp. M1 Protocol: manufacturer's recommendation. M2 protocol: manufacturer's recommendation with 35 cycles. M3 protocol: 35 cycle and longer denaturation and extension steps.

TABLE 2 Mean depth coverage and percentage of mitochondrial DNA control region (mtDNA-CR) recovered in samples used in experimental phase 1 that provides sequence results for the different methods used in library amplification (M1, M2, and M3).

	Mean depth coverage			% mtDNA-CR recovered 30×			% mtDNA-CR recovered 10×		
Sample	M1	M2	M3	M1	M2	M3	M1	M2	M3
1	191.9	230.5	315.6	96.2	34.1	71.5	96.2	84.6	90.2
4	44.2	193.5	225.6	11.0	13.6	46.8	12.8	53.7	69.4
7	2.0	160.9	583.5	0.8	32.2	82.3	7.8	60.5	96.2
15	10.2	451.5	454.0	11.0	37.7	74.6	11.0	61.6	82.5
16	0.6	1.1	5.8	0.0	0.0	7.7	0.0	0.0	11.0
17	97.8	425.9	322.4	26.7	68.7	38.4	35.6	96.2	78.0

Note: M1 protocol: manufacturer's recommendation. M2 protocol: manufacturer's recommendation with 35 cycles. M3 protocol: 35 cycle and longer denaturation and extension steps.

tion coverage of more than 30× is implemented (Table 3). If by position coverage of more than 10× is considered, only three samples analyzed with M3 (9.7%) have less than 15% of the recovered mtDNA-CR, and in 41.9% of the samples analyzed with M3, more than 85% of the mtDNA-CR was recovered (Table 3).

3.1.3 | Data authentication: percentage of damaged reads

To ascertain whether the better results obtained with the M2 and particularly with the M3 protocols are not biased by the amplification of modern human DNA contaminant, the percentage of damaged reads (calculated without duplicate reads) was computed, and the results compared with that obtained for the reference samples.

Table 4 presents the results of mean, minimum, and maximum values of the percentage of damage reads obtained for present day and 20th century reference samples, for which around 100% of mtDNA-CR was recovered. In accordance with the results for the reference samples, ancient samples are expected to present damage values higher than the maximum value reported for present-day samples. Moreover, attending to the value obtained for 20th century reference samples, the

TABLE 3 Number and percentage of samples used in phase 2 with sequence results for less than 15% and for more than 85% of the mitochondrial DNA control region (mtDNA-CR).

	Number of samples (%) with mtDNA-CR recovered ≤15%		Number of samples (%) with mtDNA-CR recovered ≥85%	
	M2	M3	M2	M3
By position coverage of more than 30×	15 (48)	8 (25.8)	3 (9.7)	7 (22.6)
By position coverage of more than 10×	11 (35.5)	3 (9.7)	8 (25.8)	13 (41.9)

Note: M2 protocol: manufacturer's recommendation with 35 cycles. M3 protocol: 35 cycle and longer denaturation and extension steps.

percentage of damaged reads would be higher than 20%. However, a small deviation can be observed if the percentage of mtDNA-CR recovered is very low, because the probability of damage is not uniform in the mtDNA-CR.

Considering the six samples that provide results with the three library protocols (Table 5), it is observed that the percentage of damaged reads does not indicate any reduction in damage with the change of protocol between M2 and M3.

If samples included in phase 2 were considered (M2 and M3, Table S1), it is evidenced that in some samples, such as 26 and 27, the recovered fraction is so low that the percentage of damage cannot be ascertained with confidence. Thus, considering samples with a percentage of recovered mtDNA-CR higher than 15% and sequenced using M2 and M3, the damage values are similar in both methods with values consistent with ancient samples (the exceptions of samples 28 and 30 will be discussed later).

3.2 | Experimental phase 3: samples sequenced with M3

3.2.1 | Percentage of genetic information recovered

Table S2 displays results for the 41 samples included in phase 3. All the libraries were considered valid for sequencing. In 31 (75.6%) and 17 samples (41.5%), respectively, more than 48% and 85% of the mtDNA-CR were recovered with a by position coverage higher than 10×. Moreover, the sequence results represent less than 15% of the mtDNA-CR in only 7 (17%) samples.

The genetic information obtained by amplicon, considering a by position coverage higher than 10×, is displayed by sample in Table S3. Figure 3 presents the percentage of samples with an mtDNA by amplicon recovery higher than 85%. Amplicons 2, 3, and 8 present the worst results, being recovered in less than 50% of the samples, whereas amplicons 1, 4, and 5 present the best results, being recovered almost totally in more than 70% of the samples.

3.2.2 | Comparison of call variant and mixed bases between in-house pipeline and GeneMarker HTS

Table S2 displays call variant and mixed bases by sample obtained with the in-house pipeline (which excludes duplicate reads) and GeneMarker HTS. Call variant was performed only for samples with a minimum mtDNA-CR recovery of 15% considering positions with more than 10 reads (calculated using data generated with the in-house pipeline). A mixed base threshold of 30% was considered.

As can be observed, there are positions that are called with the in-house pipeline and not with GeneMarker HTS or vice versa. As for fixed positions, in all cases, the discordance is related to the fact that the region of the variant was not recovered in sufficient quality and coverage depth (minimum 10 reads) implemented in the specific instrument of analysis. In this sense, the GeneMarker HTS can call more fixed variants than the in-house pipeline because the variant call was performed with duplicates. For mixed base positions, the opposite trend is observed, whereby the in-house pipeline can call more mixed base positions (14) than the GeneMarker HTS (4). The discrepancies observed are related with the fact that if no duplicates are included, the retrieved sequence represents original molecules in the sample, and the results are not biased by the fact that some molecules can be preferentially amplified during PCR. Hence, the in-house pipeline appears to be more efficient at detecting sample mixtures or contamination.

3.2.3 | Sample contamination: molecular damage and mixed bases

Considering the limits of molecular damage established for the reference samples, from the samples sequenced with M3 and with a recovery higher than 15% of the mtDNA-CR (Table S2 for detailed data), three samples (Table 6) have a lower percentage of damaged reads than those expected for ancient samples.

Regarding mixed bases (see Table S2 for detailed data), most samples have no position or only one, but there

TABLE 4 Results of mean and 95% confidence interval (CI), minimum, and maximum values of the percentage of damage reads obtained for present day and 20th century reference samples.

Reference samples	*N*	Mean (95% CI)	Minimum	Maximum
20th century	18	33.21 (29.22–37.21)	20.73	45.08
Present day	12	2.75 (2.16–3.35)	1.46	4.08

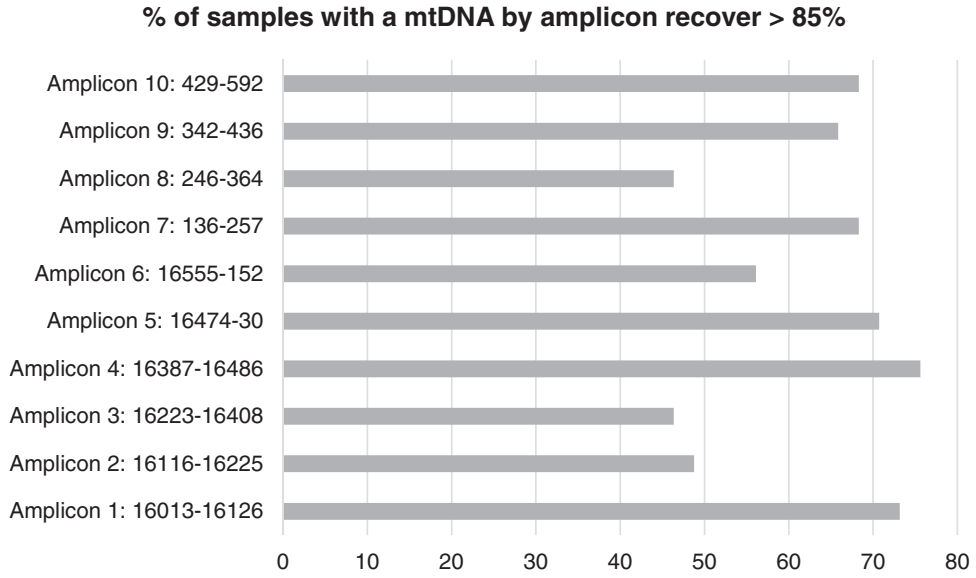

FIGURE 3 Percentage of samples with mitochondrial DNA (mtDNA) recovery higher than 85% by amplicon.

TABLE 5 Percentage of damaged reads recovered in samples used in experimental phase 1 that provide sequence results for the different methods used in library amplification (M1, M2, and M3).

Sample	M1	M2	M3
1	35.47	22.39	23.60
4	16.75[a]	20.05	19.31
7	20.45[a]	22.27	20.40
15	18.75[a]	21.65	21.63
16	20.83[a]	34.78[a]	23.86[a]
17	13.56	18.68	21.15

Note: M1 protocol: manufacturer's recommendation. M2 protocol: manufacturer's recommendation with 35 cycles. M3 protocol: 35 cycle and longer denaturation and extension steps.

[a]Experiments in which less than 15% of the mitochondrial DNA control region (mtDNA-CR) was recovered.

are two samples that present several mixed base positions (Table 6).

The five samples detailed in Table 6 do not provide authentic results, and detection was possible by combining two different strategies: the presence of molecular damage in ancient samples and the mixed base composition detected after excluding duplicate reads. Samples 19 and 21 present a high percentage of damaged reads, but the high level of mixed bases indicates a mixed profile, which is evidenced on observation of the alignment with IGV. In contrast, 28, 30, and 40 present very good alignment without mixed bases, but the percentage of damage is too low, indicating that the sample was contaminated by modern DNA and that it was preferentially recovered.

4 | DISCUSSION

Previous studies have documented the effectiveness of using NGS for genetic identification of degraded human skeletal remains [38–40]. Most of these have reported an increase in the recovery of genetic information as the NGS technique uses more sensitive and robust commercial kits.

In this study, we tested the recovery of the mtDNA-CR by NGS from a wide range of archaeological samples (5500 BP–20th century) using the PowerSeq CRM Nested System kit. The results show that it is possible to recover more than 85% of the mtDNA-CR from ancient human skeletal remains. However, some modifications to the PCR conditions were necessary to obtain successful results. We

TABLE 6 Samples with several mixed bases or with a low percentage of damaged reads.

Sample ID	Chronology and skeletal remains	Mapped reads without duplicate ($Q > 30$)	% mtDNA-CR recovered (10× positions)	Damaged reads	% Damaged read (damaged reads/mapped reads without duplicates)	No. of mixed bases in-house/GeneMarker HTS
19	5500 BP; metatarsal	796	88.40	320	40.20	4/1
21	5th BC; petrous	14 761	49.00	2932	19.86	3/1
28	16th century; phalanx	20 482	63.27	1662	8.11	0/0
30	20th century; dental pulp	9214	96.24	649	7.04	0/0
41	19th century; radius	6799	48.50	482	7.09	1/0

evaluated three protocols: M1 (manufacturer's recommendations), M2 (manufacturer's recommendations with 35 cycles), and M3 (also 35 cycles, and longer denaturation and extension steps). M3 proved to be the best protocol with the highest percentage recovery of mtDNA-CR. The DNA from aged or degraded tissue is often highly fragmented due to autolysis, bacterial degradation, and spontaneous depurination (patterns of molecular damage). This fragmentation severely reduces the efficiency of PCR. However, as in the present study, it has been demonstrated that the slight increase in the number of cycles (no more than 35 cycles), and longer denaturation and extension steps (15–60 s), enables full-length polymerization and good DNA yields [41].

In the forensic context, mtDNA-CR typing is based on the production of PCR libraries from previously amplified products of mtDNA-CR by NGS. The library is usually prepared in two steps: The first is the amplification of specific regions (HVS-I, HVS-II, or HVS-III), and the second is library preparation (addition and indexing of adapters) [42–44]. In our study, we obtained good results for the mtDNA-CR with the amplification and library preparation of 10 amplicons spanning the whole mtDNA-CR in just one PCR step, thus avoiding many steps and decreasing the risk of contamination. The results showed that the M3 protocol was more successful at retrieving the 10 amplicons than the other protocols. However, amplicons 2, 3, and 8 yield poor results in half of the samples. Amplicon 3 (spanning region from 16 223 to 16 408) is the largest, and its poor results could be partly related to its size. As for amplicons 2 and 8, both are of a similar size of around 120 bp, and their failure cannot be attributed to size. Holland et al. [19] identified potential molecular damage hotspots across the mtDNA-CR and amplicons 2 and 3 accumulate, respectively, 4 and 7 damage hotspots, and some of these are located in primer binding sites. Thus, it is possible that molecular damage could impair the number of DNA molecules and could also limit primer binding, which would produce low coverage or the absence of results for these regions. Moreover, homopolymeric C-stretch regions located at nucleotide positions 16 184–16 193 (amplicon 2) in HVI and at positions 303–315 in HVII (amplicon 8) are highly prone to present heteroplasmy produced by the insertion/deletion of cytosines [45]. The presence of homopolymeric C-stretch generates low quality reads, reducing the number of reads that pass quality filters, and the alignment of these regions is usually poor [46, 47]. Accordingly, the low performance of amplicons 2 and 8 can also be related to homopolymeric C-stretch.

Although molecular damage can impair PCR and sequencing results, its presence can be used to authenticate results obtained [24] when changing the PCR protocol. As has been demonstrated previously [19, 24], modern DNA has no damage or a low level of molecular damage, whereas ancient samples accumulate molecular damage. Our results for the percentage of molecular damage for reference samples agree with previous publications, and we were able to establish limits of damage to evaluate our results. The levels of molecular damage do not change in samples sequenced using M1, M2, and M3, and neither do they in samples analyzed for both M2 and M3, indicating that the proposed PCR conditions do not change the percentage of damaged molecules that were amplified. As we excluded PCR duplicates from the analyses, we can ascertain the proportion of original molecules that present damage and that were amplified in relation to the ones that do not present damage. As a whole, the results indicate that changes in protocol do not potentiate the amplification of non-damaged molecules.

Concerning the detection of contamination, our results suggest that a holistic approach is required to authenticate the results, and that both the number of mixed bases and the percentage of damaged reads must be implemented and only reads without duplicates should be included in the analysis.

Regarding the analysis tools used to determine the variants or haplotypes from each library generated, we found

strong concordance of variants between both GeneMarker HTS software and the in-house pipeline. However, the GeneMarker HTS software does not estimate the percentage of reads with molecular damage. This parameter should be evaluated for all highly degraded samples as it indicates and confirms the recovery of degraded DNA as opposed to possible contamination of modern DNA [19]. The mixed bases were reported in both. However, the in-house pipeline determined more variant mixtures than the GeneMarker HTS software. This could be due to the differences in the analysis workflow of the GeneMarker HTS software, as mixed bases are not reported when there is a disequilibrium between the base in forward and reverse sequence and because duplicate reads were added to the analysis. An advantage of both analysis tools is that both can mitigate sequencing errors, excluding the coordinates of the primer binding sites, avoiding overlapping regions, and providing only the informative sequence.

To summarize, the comparison of three NGS protocols analyzed in this study showed how successful the M3 protocol is at recovering the whole mtDNA-CR from highly degraded skeletal samples of different chronologies to solve maternal kinship cases in a forensic context or for population studies. There are advantages of using the *PowerSeq CRM Nested System* kit based on a single-multiplex PCR reaction as it substantially reduces the workload and decreases the risk of cross-contamination, as well as time and costs. Our freely available in-house pipeline can provide variants concordant with the forensic software. Finally, unlike the GeneMarker HTS software, it can confirm and quantify the molecular damage of degraded DNA.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of Sarah Copeland from Soft Genetics and her scientific contributions to the analysis of results. The authors also thank the reviewers for their critical observations that have helped to improve the manuscript. This research was supported by the Ministerio de Ciencia e Innovación (Ref. PGC2018-096666-B-100). DRCA is a PhD fellow from the Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) (2021 FI_B1 00064). CS and AM are members of GREAB supported by the Generalitat de Catalunya (Ref. 2017 SGR 1630; 2021 SGR 00186).

Open access funding provided by CRUE-CSIC.

CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

ORCID

Diana C. Vinueza-Espinosa https://orcid.org/0000-0001-9931-2108
Daniel R. Cuesta-Aguirre https://orcid.org/0000-0001-7483-8541
Assumpció Malgosa https://orcid.org/0000-0003-1723-3671
Cristina Santos https://orcid.org/0000-0002-7014-8014

REFERENCES

1. Rohland N, Glocke I, Aximu-petri A, Meyer M. Extraction of highly degraded DNA from ancient bones, teeth and sediments for high-throughput sequencing. Nat Protoc. 2018;13:2447–61.
2. Butler JM. Advanced topics in forensic DNA typing: methodology. 1st ed. Amsterdam: Elsevier; 2012.
3. Zavala EI, Rajagopal S, Perry GH, Kruzic I, Bašić Ž, Parsons TJ, et al. Impact of DNA degradation on massively parallel sequencing-based autosomal STR, iiSNP, and mitochondrial DNA typing systems. Int J Legal Med. 2019;133(5):1369–80.
4. Eichmann C, Parson W. 'Mitominis': multiplex PCR analysis of reduced size amplicons for compound sequence analysis of the entire mtDNA control region in highly degraded samples. Int J Legal Med. 2008;122(5):385–8.
5. Eduardoff M, Xavier C, Strobl C, Casas-Vargas A, Parson W. Optimized mtDNA control region primer extension capture analysis for forensically relevant samples and highly compromised mtDNA of different age and origin. Genes (Basel). 2017;8(10):237.
6. Alonso A, Albarrán C, Martıń P, Garcıá P, Garcıá O, de la Rúa C, et al. Multiplex–PCR of short amplicons for mtDNA sequencing from ancient DNA. Int Congr Ser. 2003;1239:585–8.
7. Gabriel MN, Huffine EF, Ryan JH, Holland MM, Parsons TJ. Improved MtDNA sequence analysis of forensic remains using a "mini-primer set" amplification strategy. J Forensic Sci. 2001;46(2):14957J.
8. Amorim A, Fernandes T, Taveira N. Mitochondrial DNA in human identification: a review. PeerJ. 2019;7:e7314.
9. Bruijns B, Tiggelaar R, Gardeniers H. Massively parallel sequencing techniques for forensics: a review. Electrophoresis. 2018;39(21):2642–54.
10. Jäger AC, Alvarez ML, Davis CP, Guzmán E, Han Y, Way L, et al. Developmental validation of the MiSeq FGx Forensic Genomics System for Targeted Next Generation Sequencing in Forensic DNA Casework and Database Laboratories. Forensic Sci Int Genet. 2017;28:52–70.
11. Diroma MA, Modi A, Lari M, Sineo L, Caramelli D, Vai S. New insights into mitochondrial DNA reconstruction and variant detection in ancient samples. Front Genet. 2021;12:619950.

12. Ta MTA, Nguyen NN, Tran DM, Nguyen TH, Vu TA, Le DT, et al. Massively parallel sequencing of human skeletal remains in Vietnam using the precision ID mtDNA control region panel on the Ion S5TM system. Int J Legal Med. 2021;135(6):2285–94.
13. Holland MM, Cave CA, Holland CA, Bille TW. Development of a quality, high throughput DNA analysis procedure for skeletal samples to assist with the identification of victims from the World Trade Center attacks. Croat Med J. 2003;44(3):264–72.
14. Ballard D, Winkler-Galicki J, Wesoły J. Massive parallel sequencing in forensics: advantages, issues, technicalities, and prospects. Int J Legal Med. 2020;134(4):1291–303.
15. Orlando L, Allaby R, Skoglund P, Der Sarkissian C, Stockhammer PW, Ávila-Arcos MC, et al. Ancient DNA analysis. Nat Rev Methods Primers. 2021;1(1):14.
16. England R, Harbison S. A review of the method and validation of the MiSeq FGxTM Forensic Genomics Solution. WIREs Forensic Sci. 2020;2(1):e1351.
17. Barrio PA, García Ó, Phillips C, Prieto L, Gusmão L, Fernández C, et al. The first GHEP-ISFG collaborative exercise on forensic applications of massively parallel sequencing. Forensic Sci Int Genet. 2020;49:102391.
18. Brandhagen MD, Just RS, Irwin JA. Validation of NGS for mitochondrial DNA casework at the FBI Laboratory. Forensic Sci Int Genet. 2020;44:102151.
19. Holland CA, McElhoe JA, Gaston-Sanchez S, Holland MM. Damage patterns observed in mtDNA control region MPS data for a range of template concentrations and when using different amplification approaches. Int J Legal Med. 2021;135(1):91–106.
20. Pilli E, Modi A, Serpico C, Achilli A, Lancioni H, Lippi B, et al. Monitoring DNA contamination in handled vs. directly excavated ancient human skeletal remains. PLoS One. 2013;8(1):e52524.
21. Cooper A, Poinar HN. Ancient DNA: do it right or not at all. Science. 2000;289:1139.
22. Spencer M, Howe CJ. Authenticity of ancient-DNA results: a statistical approach. Am J Hum Genet. 2004;75(2):240–50.
23. Danielewski M, Żuraszek J, Zielińska A, Herzig K-H, Słomski R, Walkowiak J, et al. Methodological changes in the field of paleogenetics. Genes. 2023;14:234.
24. Skoglund P, Northoff BH, Shunkov MV, Derevianko AP, Pääbo S, Krause J, et al. Separating endogenous ancient DNA from modern day contamination in a Siberian Neandertal. Proc Natl Acad Sci. 2014;111:2229–34.
25. Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, et al. Genetic analyses from ancient DNA. Annu Rev Genet. 2004;38:645–79.
26. Jónsson H, Ginolhac A, Schubert M, Johnson PLF, Orlando L. mapDamage2.0: fast approximate Bayesian estimates of ancient DNA damage parameters. Bioinformatics. 2013;29(13):1682–4.
27. Handt O, Höss M, Krings M, Pääbo S. Ancient DNA: methodological challenges. Experientia. 1994;50(6):524–9.
28. Gorden EM, Sturk-Andreaggi K, Marshall C. Repair of DNA damage caused by cytosine deamination in mitochondrial DNA of forensic case samples. Forensic Sci Int Genet. 2018;34:257–64.
29. Gilbert MTP, Willerslev E, Hansen AJ, Barnes I, Rudbeck L, Lynnerup N, et al. Distribution patterns of postmortem damage in human mitochondrial DNA. Am J Hum Genet. 2002/12/12. 2003;72(1):32–47.
30. Dabney J, Meyer M, Pääbo S. Ancient DNA damage. Cold Spring Harb Perspect Biol. 2013;5(7):a012567.
31. Espinosa DCV. Methodological challenges in ancient and forensic DNA analysis : improvements in DNA extraction and genetic characterization from human skeletal remains. Doctoral thesis. Universitat Autònoma de Barcelona; 2022. p. 254.
32. Holland MM, Pack ED, McElhoe JA. Evaluation of GeneMarker® HTS for improved alignment of mtDNA MPS data, haplotype determination, and heteroplasmy assessment. Forensic Sci Int Genet. 2017;28:90–8.
33. Vinueza-Espinosa DC, Santos C, Martínez-Labarga C, Malgosa A. Human DNA extraction from highly degraded skeletal remains: how to find a suitable method? Electrophoresis. 2020;41:2149–58.
34. Andrews S. FASTQC. A quality control tool for high throughput sequence data. FASTQC; 2010. FastQCDownload
35. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754–60.
36. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat Biotechnol. 2011;29(1):24–6.
37. Weissensteiner H, Pacher D, Kloss-Brandstätter A, Forer L, Specht G, Bandelt H-J, et al. HaploGrep 2: mitochondrial haplogroup classification in the era of high-throughput sequencing. Nucleic Acids Res. 2016;44(W1):W58–63.
38. Zupanič Pajnič I, Fattorini P. Strategy for STR typing of bones from the Second World War combining CE and NGS technology: a pilot study. Forensic Sci Int Genet. 2021;50:102401.
39. Gorden EM, Sturk-Andreaggi K, Marshall C. Capture enrichment and massively parallel sequencing for human identification. Forensic Sci Int Genet. 2021;53:102496.
40. Young JM, Higgins D, Austin JJ. Hybridization enrichment to improve forensic mitochondrial DNA analysis of highly degraded human remains. Front Ecol Evol. 2019;7:450.
41. Hedell R, Dufva C, Ansell R, Mostad P, Hedman J. Enhanced low-template DNA analysis conditions and investigation of allele dropout patterns. Forensic Sci Int Genet. 2015;14:61–75.
42. Liu L, Li Y, Li S, Hu N, He Y, Pong R, et al. Comparison of next-generation sequencing systems. J Biomed Biotechnol. 2012;2012:251364.
43. McElhoe JA, Holland MM, Makova KD, Su MSW, Paul IM, Baker CH, et al. Development and assessment of an optimized next-generation DNA sequencing approach for the mtgenome using the Illumina MiSeq. Forensic Sci Int Genet. 2014;13:20–9.
44. Yang Y, Xie B, Yan J. Application of next-generation sequencing technology in forensic science. Genomics Proteomics Bioinformatics. 2014;12(5):190–7.
45. Santos C, Sierra B, Alvarez L, Ramos A, Fernández E, Nogués R, et al. Frequency and pattern of heteroplasmy in the control region of human mitochondrial DNA. J Mol Evol. 2008;67(2):191–200.
46. Battle SL, Puiu D, Verlouw J, Broer L, Boerwinkle E, Taylor KD, et al., TOPMed mtDNA Working Group. A bioinformatics pipeline for estimating mitochondrial DNA copy number and heteroplasmy levels from whole genome sequencing data. NAR Genom Bioinform. 2022;4(2):lqac034.

47. Sturk-Andreaggi K, Parson W, Allen M, Marshall C. Impact of the sequencing method on the detection and interpretation of mitochondrial DNA length heteroplasmy. Forensic Sci Int Genet. 2020;44:102205.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Vinueza-Espinosa DC, Cuesta-Aguirre DR, Malgosa A, Santos C. Mitochondrial DNA control region typing from highly degraded skeletal remains by single-multiplex next-generation sequencing. Electrophoresis. 2023;44:1423–1434. https://doi.org/10.1002/elps.202200052