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PR7001-1593

Received: 8 May 2023 | Revised: 7 June 2023 | Accepted: 7 July 2023

DOI: 10.1002/elps.202300097

ELECTROPHORESIS

RESEARCH ARTICLE

A comparison between petrous bone and tooth, femur and tibia DNA analysis from degraded skeletal remains

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Abstract

Skeletal remains are the only biological material that remains after long periods; however, environmental conditions such as temperature, humidity, and pH affect DNA preservation, turning skeletal remains into a challenging sample for DNA laboratories. Sample selection is a key factor, and femur and tooth have been traditionally recommended as the best substrate of genetic material. Recently, petrous bone (cochlear area) has been suggested as a better option due to its DNA yield. This research aims to evaluate the efficiency of petrous bone compared to other cranium samples (tooth) and postcranial long bones (femur and tibia). A total amount of 88 samples were selected from 38 different individuals. The samples were extracted by using an organic extraction protocol, DNA quantification by Quantifiler Trio kit and amplified with GlobalFiler kit. Results show that petrous bone outperforms other bone remains in quantification data, yielding 15–30 times more DNA than the others. DNA profile data presented likeness between petrous bone and tooth regarding detected alleles; however, the amount of DNA extracted in petrous bones allowed us to obtain more informative DNA profiles with superior quality. In conclusion, petrous bone or teeth sampling is recommended if DNA typing is going to be performed with environmentally degraded skeletal remains.

KEYWORDS
degraded DNA, forensic genetics, human remains, petrous bone, skeletal remains

1 | INTRODUCTION

DNA typing from skeletal remains is a valuable resource in forensic, archaeological, and ancient DNA studies since bones and teeth are the only biological material that remains after long periods of decaying and environmental exposure [1]. The environmental conditions such as high temperature [2], humidity [3], salinity, and low pH [4] are factors that affects DNA preservation [5], resulting in molecular damage and degradation in small pieces hindering its recovery in laboratory. In this context, the use of

Abbreviations: AT, analytical threshold; CQV, coefficient of quartile variation; RFU, relative fluorescence units; ST, stochastic threshold.

ancient DNA methods could be a valuable strategy for the forensic DNA community [6].

In forensic genetics, DNA yield has been found to be different between skeletal remains, resulting in poor or high DNA profile quality; thus, sample selection is a key factor to consider. It has been traditionally established that cortical matter of long bones (femur, tibia, or fibula) and intact teeth (molar and premolars) have a higher success rate than humerus, radius, or ulna in DNA analyses [7]. A systematic review found that bone samples were more reported (89%) than teeth (11%), having these two groups of samples no differences in quantification values, however having femur more success in DNA profile obtention [8].

Nevertheless, some studies have demonstrated that the petrous portion of the temporal bone is a valuable sample source for DNA typing [9], yielding considerably more short tandem repeat (STR) markers than femurs or teeth [10], even in burned remains [11]. However, there is scant research with a high number of samples and bones from the same individual. Petrous bone success might be explained by a bone protective cover around the otic capsule and less vascularization [12] and the higher presence of osteocytes, three times compared to femur cortical bone [13]. Still, there are times when the sample cannot be chosen due to the remains conservation state or even the evidence that has been found: in anthropological contexts, cranium may be missing approximately 15% of the times, while femur and tibia are absent around 50% of the cases, being these three groups of remains fragmented 15%–20% of the times [14].

The Program of Identification of the Victims of the Spanish Civil War and Afterwar Period in Andalusia has the mission to recover and identify the human remains found in mass graves in all the Andalusian territory. In order to achieve these objectives, this laboratory is elaborating a DNA database made up by genetic profiles of the different skeletal remains recovered from archaeological interventions in mass graves, and a reference database formed by the genetic profiles of the available victims' relatives.

The research aim is to evaluate if it is possible to improve the DNA analyses results by choosing a certain bone part. To assess that, four skeletal elements were analyzed, both cranial (petrous and teeth) and postcranial (femur and tibia).

2 | MATERIALS AND METHODS

Sample preparation, DNA extraction, quantification, and amplification were performed in a low copy number DNA laboratory facility according to the recommendations given to this kind of samples [15–17]. Contamination prevention measures included room UV radiation, HEPA (High Efficiency Particle Arresting)-filtered air positive pressure, working surfaces cleaning by DNAZap (ThermoFisher), and sterilized laboratory material. Degradation index values, "ski-slope" profiles, and comparison between samples and laboratory staff profiles were used for contamination detection.

2.1 | Sample preparation

After anthropological examination, a total amount of 88 bones or teeth were received and analyzed by this laboratory, coming from mass graves of the same region in Andalusia (Southern Spain): 11 tibia, 21 femur, 22 tooth, and 34 petrous bones from 38 different individuals (see Table 1). Individuals were mostly male adults (18–50 years old). Samples degree of preservation ranged from light, hollow, fragile remains to slightly granular ones [18]. Sample selection was based on the premise of having the four different skeletal remains sample types; thus, this was not possible in every individual due to the state of decay of the remains, so at least two remains per individual were sampled.

Samples were buried in mass graves of approximately 4 m depth during 70–80 years in the South-West region of Andalusia, the southernmost territory in Spain, a region with average temperatures of 28°C and maximum temperatures of 45°C during summer, more than 2800 h of annual solar radiation (5 kW/h/m^2), minimum temperatures of 12°C in winter, and an average annual precipitation of 400–600 mm rain gauge [19], making the soil of this area slightly acidic [20]. No field data are available.

Samples exterior surface was sanded and then cut in 0.5–1 cm fragments with a Dremel rotatory tool [21]. After that, fragments were exposed to UV light in a 6 W UV cabin, each side during 10 min [22], and then grinded in a TissueLyser II (QIAGEN) under two cycles of 30 Hz for 30 s. Note that 1.0 g of skeletal remain powder was transferred to a 15-mL Falcon tube.

2.2 | DNA extraction

DNA samples were extracted following an in-house protocol based on traditional phenol-chloroform-isoamyl protocol [23]. Note that 5 mL of extraction buffer containing 4125 µL EDTA 0.5 M, 300 µL SDS 10%, 375 µL proteinase K 10 mg/mL, and 200 µL DTT 1 M was added to 1.0 g of skeletal remain powder and then incubated at 56°C overnight. After that the lysate was centrifuged at maximum revolutions for 5 min and transferred to a clean 15-mL Falcon tube. A total of 4 mL of phenol–chloroform–isoamyl (25:24:1) was added and then samples

TABLE 1 Skeletal remains samples by individual (I).

I1	I2	I3	I4	I5	I6	I7	I8	I9
Petrous	Femur Tibia	Tooth Petrous	Tooth Petrous	Tooth Petrous	Tooth Petrous	Tooth Petrous	Tooth Petrous	Femur Tibia
I10	I11	I12	I13	I14	I15	I16	I17	I18
Femur Tibia	Tooth Femur Tibia Petrous	Femur Tibia Petrous	Femur Tibia Tooth Petrous	Femur Tibia Tooth	Femur Tibia Petrous	Femur Tibia Tooth Petrous	Femur Tibia Tooth Petrous	Femur Tibia Tooth Petrous
I19	I20	I21	I22	I23	I24	I25	I26	I27
Tooth Petrous	Tooth Petrous	Tooth Petrous	Tooth Petrous	Tooth Petrous	Tooth Petrous	Tooth Petrous	Tooth Petrous	Tooth Petrous
I28	I29	I30	I31	I32	I33	I34	I35	I36
Tooth Petrous	Femur Petrous	Femur Petrous	Femur Petrous	Femur Petrous	Femur Petrous	Femur Petrous	Femur Petrous	Femur Petrous
I37	I38							
Femur Petrous	Femur Petrous							

were centrifuged at maximum revolutions for 5 min. The supernatant phase was transferred to an Amicon Ultra-4 Centrifugal Filter Unit 30 kDa (Merck KGaA) and centrifuged at 1500 *g* until most of supernatant is filtered. Eluate was discarded and two washes with 750 µL DNase- and RNase-free water were performed, each one for 20 min 1500 *g* centrifugation. Finally, DNA extracts were purified with QIAquick columns in two steps of elution, each one of 37.5 µL, obtaining a final volume of 75 µL [24].

2.3 | DNA quantification

DNA extracts were quantified with the Quantifiler Trio DNA Quantification Kit (ThermoFisher) following the manufacturer's recommendations [25]: two 2 µL replicates of each sample, five standards, and a non template control (NTC) were amplified in a QuantStudio 5 (ThermoFisher).

Four parameters were analyzed to assess DNA success recovery from the different human skeletal remains: quantity mean of the human small autosomal target (80 bp), quantity mean of the human large autosomal target (214 bp), human male target (75 bp), and degradation index (calculated as the ratio of small target and large target).

2.4 | DNA amplification and visualization

DNA extracts were amplified with GlobalFiler PCR Amplification Kit following the manufacturer's procedure [26]. Maximum volume of DNA extract (15 µL) was added to the reaction to a final volume of 25 µL and amplified in a 29 cycles reaction. Fragments were visualized in a 3500 Applied Biosystems Genetic Analyzer following manufacturer's parameters. Data were analyzed using GeneMapper ID-X 1.6. In order to assess the number of detected alleles, an analytical threshold of 50 RFU and stochastic threshold of 365 RFU (both determined after internal validation) were established.

Statistical analyses (mean, Shapiro–Wilk normality test, coefficient of quartile variation [CQV], and nonparametric tests, one-way analysis of variance and Dwass-Steel-Critchlow-Fligner [DSCF] pairwise comparison) were performed with Jamovi Version 2.2.5 [27].

Four parameters were analyzed: number of alleles detected above the analytical threshold (50 RFU), number of alleles detected above the stochastic threshold (365 RFU), average RFU, and number of reportable markers (heterozygous markers with peak hight above the analytical threshold and homozygous markers with peak hight above the stochastic threshold). Yindel, DYS391, and Amelogenin markers were excluded, so only STR informative alleles were considered in statistical analyses.

3 | RESULTS AND DISCUSSION

3.1 | DNA quantification

DNA quantification results are shown in Table 2; both average and coefficient of quartile variation (CQV), a robust dispersion parameter for nonparametric distributions . Boxplots of each target by skeletal sample are shown in Figure 1.

TABLE 2 DNA quantification results (both mean and coefficient of quartile variation) by skeletal remain sample type.

	Femur	Tibia	Tooth	Petrous
Small target (ng/μL)	0.022	0.040	0.051	0.723
CQV	0.701	0.745	0.847	0.433
Large target (ng/μL)	0.004	0.010	0.009	0.031
CQV	0.831	0.634	0.879	0.858
Male target (ng/μL)	0.023	0.028	0.047	0.560
CQV	0.651	0.464	0.896	0.562
Degradation index	7	10	12	81
CQV	0.326	0.594	0.380	0.735

Abbreviation: CQV, coefficient of quartile variation.

Petrous bone yielded the highest mean of human small autosomal target with 0.723 ng/μL, followed by tooth (0.051 ng/μL), tibia (0.040 ng/μL), and femur (0.022 ng/μL). Petrous bone reached quite high values, ranging from 0.009 to 1.824 ng/μL. Statistically significant differences were found between femur, tibia, tooth, and petrous (p-value < 0.001) in a DSCF pairwise comparison at a 95% confidence level.

Grouping samples by small DNA quantity, only 6% of the analyzed petrous bone yielded less than 50 pg DNA, while 90% of femur yielded less than that amount of DNA (82% in the case of tibia and 59% in the case of tooth), being petrous bone the only kind of sample which yielded 0.5–1 ng/μL (41% of analyzed samples) and more than 1 ng/μL (24%).

Large human autosomal target was missed by only one bone (petrous), which also achieved the highest average value (0.031 ng/μL), followed by tibia (0.010 ng/μL), tooth (0.009 ng/μL), and femur (0.004 ng/μL). The maximum was reached by petrous bone, ranging from 0.0006 to 0.176 ng/μL. There were statistically significant differences between femur and petrous (p-value = 0.003), and tibia and petrous (p-value = 0.016).

The highest degradation index values were yielded by petrous bone (81), followed by tooth (12), tibia (10) and femur (7). It must be noted that petrous bone gives much higher values of degradation index, ranging from 10 to even 100−200. However, this does not mean that DNA obtained from petrous bone is more degraded than the other skeletal remains, because if more small fragments are recovered and they are naturally degraded, the largest degradation index they will have. There was statistically significative difference between petrous and the other bone samples (p-value < 0.001).

In summary, petrous bone outperforms tooth and long bones in DNA quantification, obtaining more both small and large target, as it has been previously indicated by similar research studies in ancient DNA [6, 28, 29]. That means obtaining 15−30 times more DNA than with other samples with the same DNA extraction protocol.

In Figure 2, small human target is shown by skeletal remain type and individual, so it can be observed how petrous bone outperforms other sample types in terms of DNA quantification, yielding around 30 times more small DNA fragments than femur, and approximately 18 times more DNA than tooth within the same individual, whereas tooth yielded 10 times more DNA than femur or tibia.

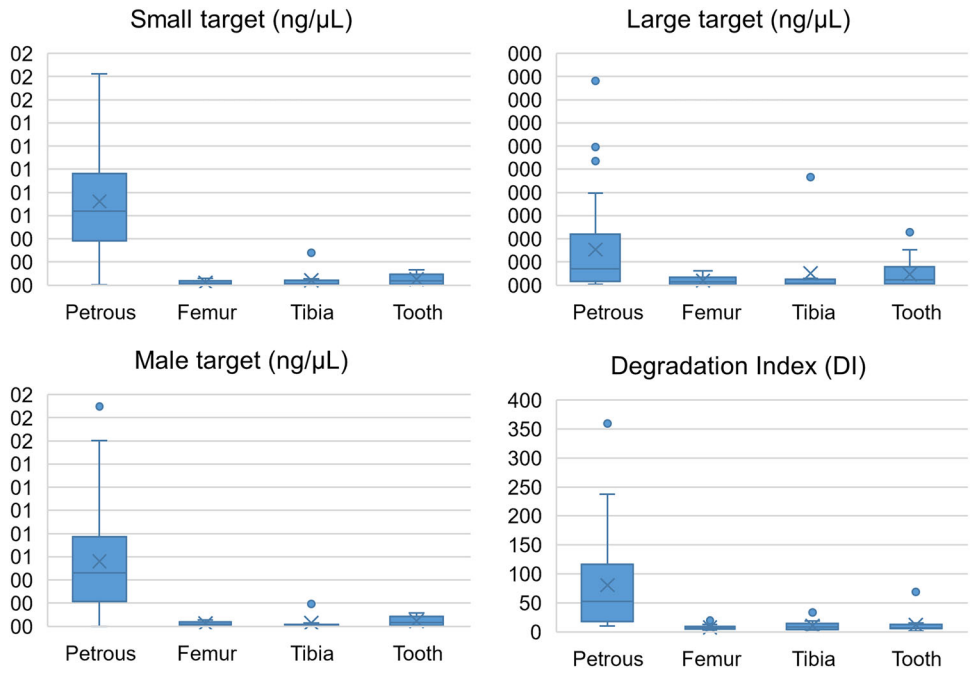

FIGURE 1 Boxplots of quantification data: Small, large, and male target, and degradation index by skeletal remain sample.

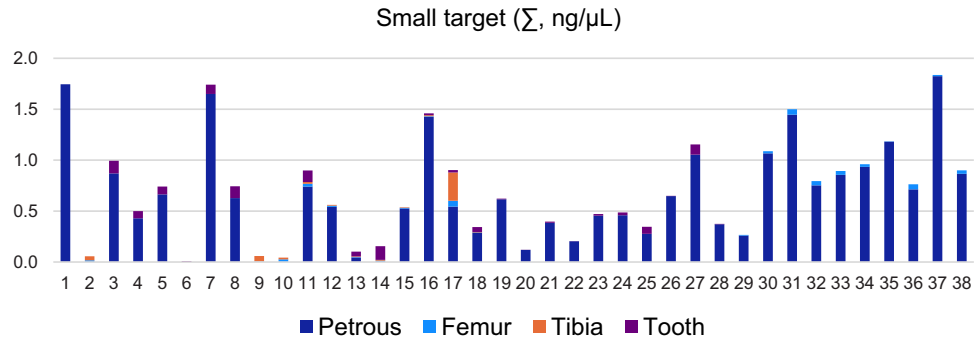

FIGURE 2 Small human autosomal target (ng/μL) detected by individual. In all cases when a petrous bone sample was available, it outperformed every other kind of sample.

Femur and tibia yielded roughly the same amount of DNA within the same individual; however, there were individuals in which tibia yielded about three times more small DNA fragments than femur.

Revisiting scientific literature, a systematic review found that tibia obtained higher DNA yield in literature, followed by teeth, both with better performance in terms of DNA quantification than femur [8]. Petrous bone has been pointed out in ancient DNA research as the remain with the largest amount of endogenous DNA quantity, with higher C→T damage rate and smaller mitochondrial DNA/nuclear DNA ratio, and high sample to sample variation [28]. In forensic literature, larger DNA quantities have been obtained from long bones [30], such as tibia [8, 31, 32], while other studies obtained better yields from petrous bone rather than from femur or teeth [10, 29, 33–36]. Petrous bone generally yields high DNA quantities and low degradation index [35, 37]. Comparing femur, tibia, and teeth, femur has been found better than teeth in terms of STR typing success, while teeth achieved better genetic profiles than tibia [7]; however, there are times when femur, tibia, and teeth are reported to have better yields than the others.

Our study supports the idea that higher DNA yields are obtained from petrous bone, whereas this kind of remain shows high variability in terms of degradation index (as stated before [38]). Nevertheless, there is a certain variability among the different studies that can be found in literature that may be explained by the sampling technique, as it has been stated that DNA is not evenly distributed around the skeletal remain [39, 40].

3.2 | Genetic profiles

Genetic profiles data (both mean and coefficient of quartile variation) are presented in Table 3, and boxplots of each parameter by sample type are shown in Figure 3.

TABLE 3 Mean and coefficient of quartile variation (CQV) of the number of detected alleles higher than analytical threshold (>AT), number of detected alleles above stochastic threshold (>ST), relative fluorescence units (RFU), and reportable loci by sample type.

	Femur	Tibia	Tooth	Petrous
Alleles > AT	24	23	30	29
CQV	0.278	0.321	0.197	0.156
Alleles > ST	12	12	19	20
CQV	0.628	0.714	0.590	0.136
RFU	744	1252	2278	5278
CQV	0.620	0.714	0.839	0.250
Reportable loci	10	10	13	15
CQV	0.632	0.565	0.505	0.172

The maximum average value of detected alleles above 50 RFU was observed in tooth (30), followed by petrous bone (29), femur (24), and tibia (23). Filtering by stochastic threshold, petrous bone was the best with an average of 20 alleles, followed by tooth (19) and femur and tibia (12). Petrous bone had the highest RFU data with an average of 5278, followed by tooth (2278), tibia (1252), and femur (744). The type of skeletal remain with the highest average number of reportable markers was petrous bone with 15, being tooth the second (13) and tibia and femur the thirds (10). Finally, a success ratio percentage, based on the number of reportable profiles obtained divided by the total amount of profiles obtained with that bone, makes petrous bone as the most successful (82%), tooth the second one (59%), and femur the third (52%), being tibia (36%) the remains with the least success rate. Statistically significative differences were obtained with petrous bone and femur alleles detected above stochastic threshold (p-value = 0.003) and reportable markers (p-value = 0.021), and between petrous and the other bone samples in average RFU (p-value < 0.001).

Comparing data among skeletal remains of the same individual in Figure 4, a certain variability can be observed

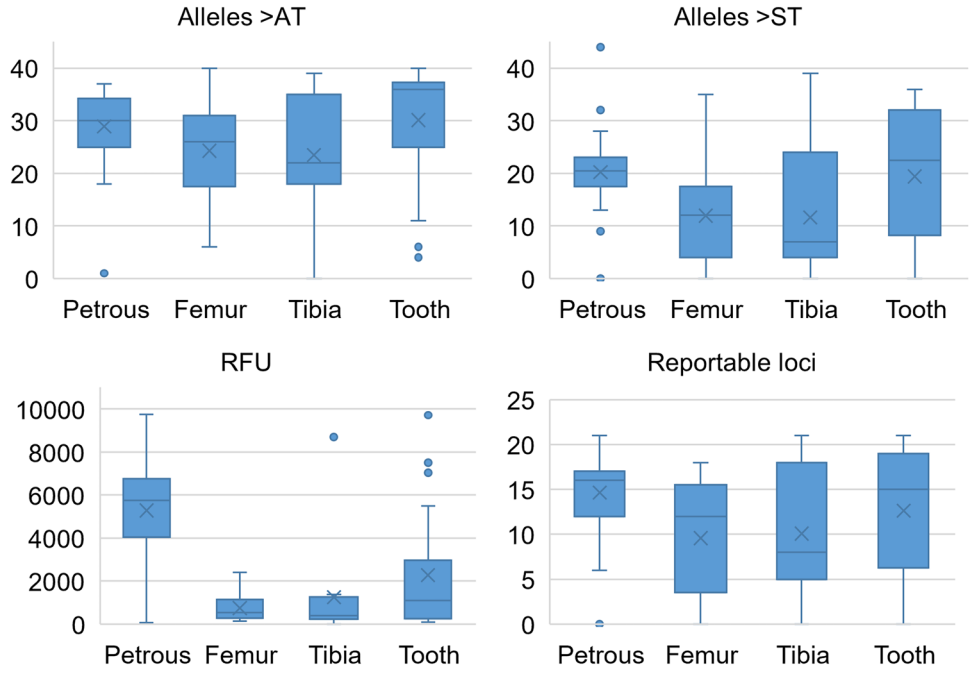

FIGURE 3 Boxplots of the number of detected alleles above analytical threshold (>AT), above stochastic threshold (>ST), average relative fluorescence units (RFU), and reportable loci by sample type.

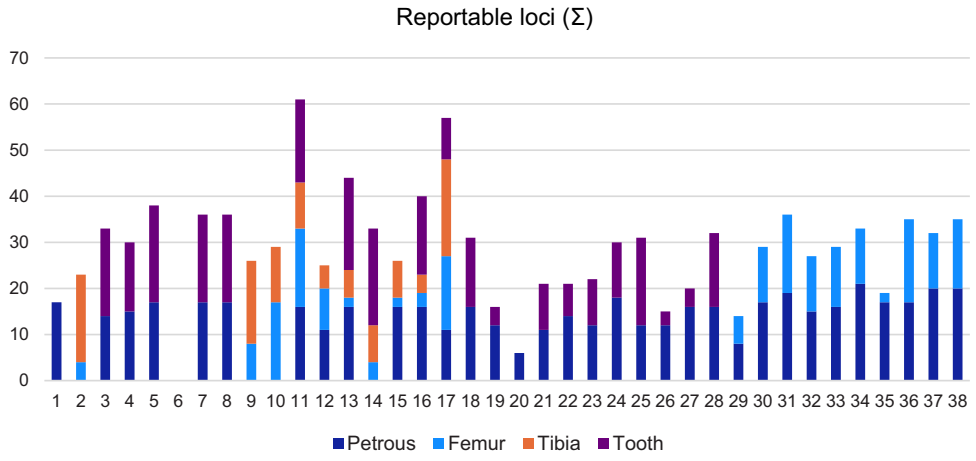

FIGURE 4 Number of reportable loci yielded by skeletal remain sample type.

since the bone that outperforms the others may be the one with less yield in other case. Both petrous and tooth outperform femur and tibia. Petrous bone is not always the bone sample that recovers the largest number of alleles, however having 15–30 times more DNA quantity than tooth, nevertheless many more alleles are recovered in some cases with dental elements, which may be explained as a better preservation of DNA in tooth than in petrous bone. Still, the main goal of obtaining an informative genetic profile from human remains cannot be overlooked, and here is where petrous bone surpasses all the other kinds of sample because if much more DNA is obtained, more amplification product is produced, leading to high RFU alleles, which in the end translates into the most informative DNA profiles. Individual 18 is a paradigmatic example of this situation: no profile was obtained from femur nor with tibia but reportable profile was achieved from tooth and petrous bone.

Discrepancies between the quantification results by commercial qPCR kits and the obtained genetic profile have already been discussed [41] when it comes to this kind of challenging samples, not only in autosomal STRs

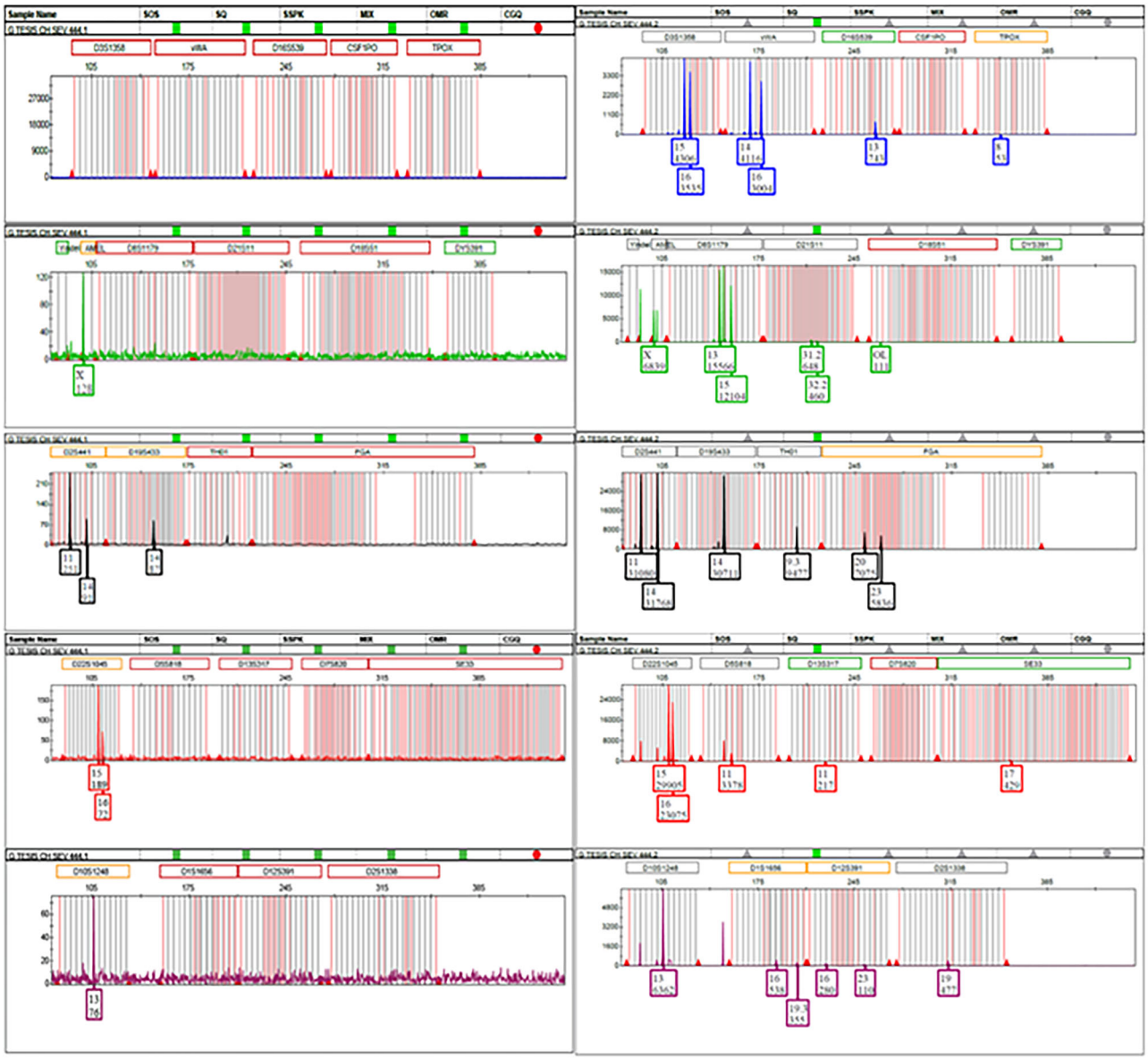

FIGURE 5 Two profiles from the same individual generated from two different skeletal remains: femur (left) and petrous bone (right).

analyses but also in mitochondrial DNA approaches [42]. As seen in quantification data discussion, long bones samples in general [30], and femur samples in particular [7, 8, 43], have been reported as the sample type thar yields the best DNA profiles. Teeth samples achieved good results taking into account the low (compared to petrous bone) DNA yields previously assessed by qPCR, as it has been stated [37]. Both teeth and petrous bone has been established by ancient DNA studies as good substrates [28]. Nevertheless, there are research examples in which teeth produced better DNA profiles [44, 45], even from samples that were buried in similar time–space conditions [31], and there are other examples in scientific literature in which petrous bone yields better DNA profiles than other skeletal sample types [10, 12, 35, 36]. Still, long bones and teeth sampling is the main recommendation from internationally renowned laboratories such as the International Commission on Missing Persons (ICMP) [46].

Regarding the DNA extraction technique, cited literature use different approaches (silica in suspension, silica columns, automated extraction, or organic extraction). In this research, organic extraction protocol was used since after a comparison of the different techniques, organic extraction showed the highest DNA yield with our human remains samples [24].

Our results back the thesis that better results are obtained from petrous bone and teeth, sample types that achieved the highest number of alleles with peak height higher than both analytical and stochastic threshold, achieving petrous bone twice the RFU obtained by

teeth (and these, in turn, twice the obtained RFU by long bones). Petrous bone outperforms teeth by the number of reportable markers; in fact, 91% of the analyzed petrous bone yielded more than 10 reportable loci, whereas only 68% of the analyzed teeth yielded reportable profiles based on that threshold (52% in the case of femur and 45% in the case of tibia), suggesting more reliability when petrous bone is analyzed.

In summary, both teeth and petrous bone produced the STR profiles with the highest number of successfully amplified alleles over lower long bones, giving petrous bone more reportable profiles than the other skeletal elements, as it has noted by previous literature [47]. Furthermore, petrous bone offers more stability since little variation in the number of detectable alleles or successfully typed remains is observed among samples. In addition, the fact that with petrous bone much more DNA can be obtained turns this sample to be highly valuable for next-generation sequencing because the small amplicon size is generated in these platforms [48]. Another advantage of petrous bone over tooth was observed during sample preparation, obtaining 2–5 g of bone powder from it, whereas only 0.5–1.5 g teeth powder is generally obtained. In addition, it is easily sampled in young individuals if skull joints are open. Nevertheless, cranium is not always available or well preserved, depending on the burial conditions, and there are scenarios such as coup de grace in which anthropologists have reported that petrous bone integrity is severely affected. Moreover, ethical issues arise when sampling petrous about the destruction of archaeological material or the fact that skulls are treated differently in some mortuary contexts [49].

As an example, a comparison of two genetic profiles is shown in Figure 5 in which the improvement in genetic profiles is observed between two different skeletal remains of the same individual. Not only more reportable alleles were achieved, but also even spectral pull-ups were detected because of the high amount of DNA recovered. In this way, DNA analysis from petrous bone makes necessary to adjust the input DNA volume in order to follow the commercial kit optimal input range so this kind of artifacts does not unfold, and as a matter of fact, profiles generated with petrous bone required less interpretation work, reducing analysis time per sample.

The aim of this work was to evaluate the efficiency of the four main type of human remains pointed out by scientific literature as the most successful for DNA typing by both qPCR and genetic profiles (regarding alleles quality control criteria) data. Furthermore, this research relies on the performance evaluation of these kinds of samples, observed as specially challenging due to the extreme conditions they have been.

4 | CONCLUDING REMARKS

Femur, tibia, teeth, and, more recently, petrous bone have been found by scientific literature as the most successful samples for DNA analysis; however, research differs in which one yields more DNA due to nonuniform DNA distribution across the sample, individual differences, environmental factors, and sampling technique.

In accordance with previous research, our study found that petrous bone yields much more DNA than tooth, femur, or tibia; however, approximately the same number of alleles are obtained with petrous bone and teeth. The former achieves more reportable markers than the latter, leading to the obtention of more valuable genetic information.

In conclusion, both petrous bone and teeth sampling is strongly recommended when DNA analysis from critically degraded human remains is practiced. This insight has allowed us to improve the success ratio in remains that are currently being analyzed.

ACKNOWLEDGMENTS

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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 from the corresponding author upon reasonable request.

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How to cite this article: Haarkötter C, Vinueza-Espinosa DC, Gálvez X, Saiz M, Medina-Lozano MI, Lorente JA, et al. A comparison between petrous bone and tooth, femur and tibia DNA analysis from degraded skeletal remains. Electrophoresis. 2023;1–10. https://doi.org/10.1002/elps.202300097