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Comparative analysis of DNA yields from different cranial bones in skeletonized remains: two forensic case studies

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Abstract: Bones and teeth are robust DNA sources for the analysis of skeletonized remains in forensic identification. Often, limited evidentiary items are available for forensic investigations, necessitating bone element selection for DNA analysis. The aim of the present study was to compare the DNA yield of five cranial bones (frontal, parietal, sphenoid, temporal, and occipital) recovered from two individuals (cases A and B) using three analytical approaches: human genomic DNA (gDNA) quantification, mitochondrial DNA (mtDNA) Sanger sequencing, and short tandem repeat (STR) typing. gDNA extracted from a specific amount of decalcified bone using the Prepfiler^{TM} Forensic DNA Extraction Kit was quantified using the Quantifiler^{TM} Trio DNA Quantification Kit, followed by PCR amplification of small HLA-DRA. mtDNA Sanger sequencing was performed, and the DNA extracted from the samples was amplified using the GlobalFiler^{TM} PCR Amplification Kit to compare STR typing efficiency. The three DNA analysis methods invariably identified the frontal bone as the foremost DNA source among the cranial bones. Although the samples were collected from only two cases, we believe that our findings could facilitate the effective selection of cranial bones for DNA analysis under resource-limited conditions.

Key words: Forensic identification, Cranial bones, DNA quantification, Mitochondrial DNA analysis, STR typing

Introduction

Bones and teeth are considered robust and reliable DNA sources owing to their extensive mineralization, which represents a mechanical barrier that protects DNA molecules from post-mortem diagenesis.[1,2] However, DNA typing from skeletonized remains can be challenging owing to various factors, including the duration of the post-mortem interval (PMI), the terrestrial environment from which the bones are

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recovered, and the types of skeletal elements available for analysis.[3] Conventionally, dense cortical weight-bearing bones (femur, tibia, and humerus) are more suitable for DNA profiling.[4,5] Nevertheless, these bones cannot always be utilized for DNA identification under resource-limited conditions. Studies reporting ancient DNA testing success rates using skeletal elements have facilitated the selection of appropriate skeletonized elements for effective DNA extraction.[6] In the skull, teeth are the preferred DNA sources for DNA fingerprinting[7,8]; Leney[6] reported that the mandible is associated with a relatively high success rate for DNA testing. However, the cranial bones are occasionally the only skeletal elements available for forensic analysis owing to disarticulation from the body or the absence of the mandible and teeth.[9,10] Forensic identification may require the simultaneous use of anthropological and anatomical approaches with DNA typing. Therefore, minimizing sample loss and selecting a suitable bone element for DNA analysis are crucial.[11]

Nonetheless, comparative studies on DNA yields from cranial bones are limited. In 2009, Edson *et al.*[12] compared cranial fragments for mitochondrial DNA (mtDNA) sequencing. However, the success rates of mtDNA profiling can differ from those of short tandem repeat (STR) typing, which is the current gold standard in forensic identification.[9] Although Mundorff and Davoren[3] cataloged DNA yields extracted from various bone samples, including four cranial bones (frontal, parietal, temporal, and occipital) recovered from skeletonized remains with a minimum PMI, they also suggested that the sample ranking could vary based on taphonomic conditions. In the present study, we surveyed five cranial bones (frontal, parietal, sphenoid, temporal, and occipital) collected from the skeletal remains of two unidentified individuals. These five cranial bones were chosen due to their accessibility for sampling, while also minimizing potential damage to the skull during the sampling process. The samples were retrieved from different sites with varying environmental conditions and subjected to forensic DNA analysis. Specifically, we compared the DNA yields of five cranial bones via gDNA quantification, mtDNA sequence analysis, and STR typing.

2. Experimental

2.1. Samples

The first skeletonized cranium, designated “Case A” (*Fig.* 1A), was discovered on a hill in Gyeongsangnam-do, Republic of Korea. The mandible and teeth were absent, and no other bones were unearthed nearby. The DNA profile of this cranium was eventually matched with that of a body found 4 years earlier. At that time, some of the remains of the body had been dispersed, and no skull was located. Therefore, even though the precise time of death was unknown, the PMI was estimated to be at least 4 years.

The second skeletonized cranium, labeled “Case B” (*Fig.* 1B), was exhumed from beneath a tree on a forest road in a public park in the province of Gyeongsangbuk-do. Although the PMI of this skeletal

Fig. 1. Bone sampling and photographs of the crania subjected to forensic examination. Photographs of (A) Case A and (B) Case B crania. (C) Five cranial bones cut into pieces for sampling. Fr, frontal; Pa, parietal; Sp, sphenoid; Te, temporal; Oc, occipital.

evidence remains undetermined, it had probably been buried for more than one decade. The mandible was absent, but three teeth were found attached to the cranium. Therefore, the two crania were recovered under different conditions; the first was discovered in an exposed condition, whereas the second was buried in the ground.

The use of the samples in this study was approved by the Institutional Review Board of the National Forensic Service of the Republic of Korea (Approval number: 906-220421-BR-007-02).

2.2. Sampling of the cranial bones and DNA extraction

The five cranial bones, namely the frontal, parietal, sphenoid, temporal, and occipital bones, were segmented into approximately 2 cm × 2 cm or 2 cm × 3 cm pieces (*Fig.* 1C). Each bone fragment was cleaned by removing surface impurities using a grinder. This was followed by rinsing three times with distilled water. Subsequently, the samples were treated with 5.5 % hypochlorite for 1 min, exhaustively rinsed with distilled water, washed with 70 % ethanol, and finally rinsed three times with distilled water. The bones were decalcified via a 72-h incubation in 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0) at 56 °C. Thereafter, the soft decalcified samples were minced, and 0.6 g samples of the bone were transferred into 2.0 mL Safe-Lock microcentrifuge tubes (Eppendorf, Hamburg, Germany) for cell lysis.

The prepared sample components were lysed in 600 L of a lysis buffer comprising 500 mL of ATL Buffer (Qiagen, Hilden, Germany), 50 mL of 1 M dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA), and 50 mL Proteinase K solution (Qiagen), via constant shaking for 12 h at 56 °C. Thereafter, the lysate was aliquoted (200 mL each) into three sterilized tubes for replica tests. DNA was then extracted using the Prepfiler™ Forensic DNA Extraction Kit (Applied Biosystems, Foster City, CA, USA), as per the manufacturer's instructions, and eluted in a final volume of 100 mL.

2.3. Quantitative analysis

The human DNA content in the eluted DNA samples was determined using the Quantifiler™ Trio Quantification Kit (Applied Biosystems), as per the manufacturer's instructions. The cycle threshold (Ct) value of the internal positive control (IPC) and the concentrations of large autosomal (LA) and small autosomal (SA) targets were determined. Further, the degradation index (DI) of the eluted DNA was calculated by dividing the SA concentration by the LA concentration.

The PCR amplification of small HLA-DRA (HLA class II histocompatibility antigen, DR alpha chain) targets was performed to evaluate the human gDNA concentration. The 25 mL PCR mixture for HLA-DRA comprised 1 mL template DNA, 2.5 mL Gold ST*R 10× buffer (Promega, Madison, WI, USA), 2.5 U Eagle Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), and 0.4 mM each of the forward and reverse primers (*Table* 1). DNA amplification was conducted using a GeneAmp® PCR System

Table 1. Primers used for human-specific gDNA PCR and mitochondrial DNA PCR

Primer		Sequence (5'–3')	Tm (°C)	Expected size (bp) / Range
HLA-DRA	F	ACTCCGATCACCAATGGTACCT	60.9	89[c]
	R	ACACAAAGAGCTGA GCAACTACT	60.7	
HV1[a]	F15971	TTAACTCCACCATTAGCACC	56.4	16051–16365[d]
	R16391	GAGGATGGTGGTCAAGGGAC	62.5	
HV2 [b]	F15	CACCCTATTAACCACTCACG	58.4	71–408[d]
	R484	TGAGATTAGTAGTATGGGAG	54.3	

[a] used for sequencing and real-time PCR with iQ™ SYBR® Green Supermix kit (BIO-RAD, Hercules, CA, USA).

[b] used for sequencing.

[c] expected size

[d] interpretation range

9700 Thermal Cycler (Applied Biosystems) while maintaining the following conditions: 96 °C for 15 min; followed by 33 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 7 min. The amplified PCR products (4 mL) were separated on a 2 % agarose gel and visualized using the UVP GelSolo image analyzer (Analytik Biosolutions, Jena, Germany). The intensity of the products was then analyzed using ImageJ software v.1.8.0 (https://imagej.nih.gov).

2.4. MtDNA sequence analysis

MtDNA was amplified for mtDNA sequencing using a 25 mL PCR mixture comprising 5 mL template DNA, 2.5 mL Gold ST*R 10× buffer, 2.5 U Eagle Taq DNA polymerase, and 0.4 mM each of the forward and reverse primers of the hypervariable regions (HV1 and HV2). *Table* 1 lists the primers utilized in this study. MtDNA was amplified using a GeneAmp® PCR System 9700 Thermal Cycler, while maintaining the following conditions: 96 °C for 15 min; followed by 35 cycles at 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 7 min. The PCR products were purified using an ExoSAP-IT™ (Applied Biosystems), and Sanger sequencing was performed using the BigDye™ Terminator v3.1 Cycle sequencing kit (Applied Biosystems), as per the manufacturer's instructions. The sequencing results were analyzed using a 3500 × L Genetic Analyzer and SeqScape® Software 3 (Applied Biosystems). Further, when the sample sequence was efficaciously assessed within the interpretation range using SeqScape® Software 3, the sample was deemed a "success." The samples were tested in triplicate, and the average success rates were calculated.

The samples that failed the sequencing analysis were subjected to quantitative real-time PCR and quantitative mtDNA differentiation for verification. Real-time PCR was performed using the iQ™ SYBR® Green Supermix kit (BIO-RAD, Hercules, CA, USA) on a C1000 Touch Thermal Cycler (BIO-RAD). The 25-mL reaction mixture comprised 1 mL template DNA, 10 µL 2× iQ™ SYBR® Green Supermix, and 0.5 mM each of the forward and reverse primers (*Table* 1). The real-time PCR conditions were maintained as follows: initial denaturation at 95 °C for 3 min, 40 cycles of denaturation at 94 °C for 15 s and annealing and extension at 56 °C for 30 s, and one cycle of a melt-curve reaction at 55–95 °C (in 0.5 °C increments) for 10–30 s. The commercial 2800 M control DNA (Promega) was included as a positive control in the quantification step.

2.5. STR typing

To compare STR typing efficiency, DNA extracted from the samples was amplified using a GlobalFiler™ PCR Amplification Kit (Applied Biosystems). For PCR, the input DNA quantity was normalized to 0.5 ng based on the SA concentration. When the DNA concentration was < 0.04 ng/mL, PCR was performed using the maximum DNA volume (15 mL). All 31 cycles of PCR were conducted in the GeneAmp® PCR System 9700 Thermal Cycler. The obtained PCR products were then resolved and confirmed using a 3500 × L Genetic Analyzer (Applied Biosystems) and analyzed using GeneMapper® ID v.1.4 software (Applied Biosystems). The analytical threshold was established at 100 relative fluorescence units, and the called alleles were counted in triplicate sets.

Amelogenin, DYS391, and Y-indel were excluded from the present study. The intra-peak balance of the heterozygous loci was computed using samples that displayed at least three detected heterozygous peaks. The height ratio within the locus was calculated by dividing the lower peak height by the higher peak height of the heterozygous peaks.

3. Results and Discussion

3.1. DNA concentration

We employed three analytical strategies, namely gDNA quantification, mtDNA analysis, and STR typing, to compare DNA yields from five cranial bones recovered from the skeletonized remains of two forensic cases. We estimated the DNA concentrations in the cranial samples obtained from both cases (*Table* 2) using the Quantifiler™ Trio Quantification Kit, which facilitates the quantification of total human

Table 2. Human gDNA concentration in cranial bones

Case	Bones	IPC (Ct)		LA conc. (pg/μL)		SA conc. (pg/μL)		DI	
A	Frontal	29.3	± 1.1	2,519.9	± 292.6	3,904.5	± 537.5	1.6	± 0.1
	Parietal	29.3	± 1.0	419.5	±73.6	765.0	± 122.7	1.9	± 0.4
	Sphenoid	28.7	± 0.4	ND		7.1	± 1.0	NA	
	Temporal	28.6	± 0.2	7.1	± 1.0	16.0	± 4.0	2.4	± 1.0
	Occipital	28.8	± 0.0	0.0		0.6	± 0.2	NA	
B	Frontal	27.0	± 0.4	ND		80.8	± 19.3	NA	
	Parietal	27.7	± 0.2	ND		0.4	± 0.1	NA	
	Sphenoid	28.0	± 0.3	ND		0.7	± 0.3	NA	
	Temporal	27.1	± 0.2	ND		0.3	± 0.1	NA	
	Occipital	29.4	± 0.8	ND		0.2	± 0.1	NA	

All data are presented as the mean ± standard deviation.

IPC: internal positive control; LA conc.: large autosomal target DNA concentration; SA conc.: small autosomal target DNA concentration; DI: degradation index; ND: Not determined, due to unreadable values from very low signal; NA: Not applicable, as DI could not be calculated when LA was ND.

gDNA and consists of SA (amplicon length: 80 bases) and LA (amplicon length: 214 bases) targets.[13] For Case A, the SA target concentration was highest in the frontal bone, followed by the parietal, temporal, sphenoid, and occipital bones. Notably, the DNA concentration in the frontal sample was 5-fold higher than that in the parietal sample, and despite belonging to the same cranium, the five bones displayed striking variations in DNA concentrations. In Case B, the DNA concentration was highest in the frontal bone; conversely, the other four had comparable concentrations, with an average of 0.4 pg/μL, which was approximately 1/200th that observed for the frontal bone. In both instances, the frontal and occipital bones consistently exhibited the highest and lowest DNA concentrations, respectively.

The LA target concentration acts as an indicator of DNA degradation, which is primarily determined by correlating SA and LA target concentrations. In Case A, the LA target concentration in the sphenoid and occipital bones could not be determined, and the average DI of the other three bones was approximately 1.9, suggesting slight DNA degradation. [13] Conversely, in Case B, the LA target in all cranial bones could not be determined. The IPC values estimated in the present study indicated that the low and undetermined DNA quantity corresponding to the LA target could be primarily attributed to DNA degradation.

The quantitative results were verified by amplifying various human-specific HLA-DRA target (89 bp) regions (*Fig*. 2). The PCR products were separated using an agarose gel, and the relative amplification intensity levels were computed and compared. We observed that the intensity levels were consistent with the DNA concentrations based on assessment using the Quantifiler™ Trio Quantification kit. Thus, the frontal bone had the highest DNA quantity among the cranial bones obtained from both cases.

3.2. MtDNA analysis

In Case A, all samples displayed a 100 % success rate. For Case B, only the frontal bone exhibited a 100 % success rate. The analysis involving the parietal, sphenoid, temporal, and occipital bones failed.

To confirm the mtDNA quantity in Case B samples, real-time PCR was conducted using the iQ™ SYBR® Green Supermix kit with HV1 primers. The results obtained showed successful amplification only for frontal bone in *Fig*. 3. The quantification values for the parietal, sphenoid, temporal, and occipital bones could not be determined. The results of real-time PCR corroborated the mtDNA sequencing failure using the samples. The average Ct value for the frontal bone was 31.9, whereas that for the positive control (100 pg of 2800 M) was 19.6. As results, mtDNA sequencing did not reveal notable variations

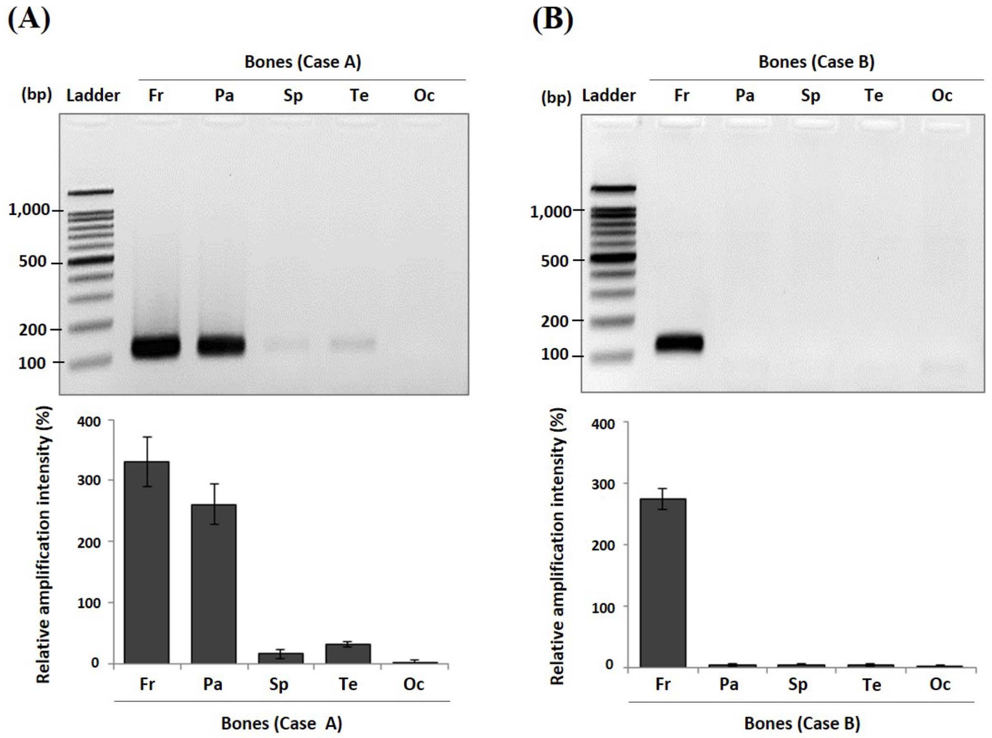

Fig. 2. Amplified PCR products of HLA-DRA targets (89 bp) from the cranial bones of (A) Case A and (B) Case B. A photograph of the PCR products separated using a 2% agarose gel (upper) and intensity of the amplified PCR product measured using ImageJ software (lower) are shown. Fr, frontal; Pa, parietal; Sp, sphenoid; Te, temporal; Oc, occipital.

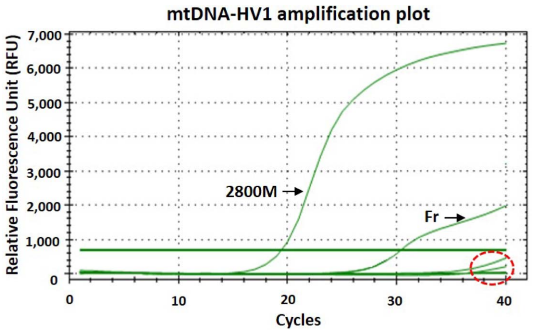

Fig. 3. Mitochondrial DNA quantification based on the HV1 regions from the cranial bones of Case B using the iQ™ SYBR® Green Supermix kit (BIO-RAD, Hercules, CA, USA); 2800M (100 pg) was used as a positive control. The red dotted circles indicate the parietal, sphenoid, temporal, and occipital bones. Fr, frontal.

between the samples in Case A. However, in Case B, the frontal bone was identified as the most suitable cranial bone for Sanger sequencing.

3.3. STR typing

STR typing efficiency was evaluated by counting called alleles (*Fig*. 4). The input DNA volume for STR amplification was adjusted based on the SA target concentration. For Case A, the average numbers of called alleles in the frontal, parietal, sphenoid, temporal, and occipital bones were 36, 36, 34.7, 35.7, and 12.3, respectively. In Case B, these was 29 for the frontal bone, and less than 5 for the parietal, sphenoid, temporal, and occipital bones. Subsequently, the intra-peak balance of the heterozygous loci was calculated. In Case A, the average peak height ratios (± standard deviation) in the frontal, parietal, sphenoid, and temporal bones were 0.84 (± 0.11), 0.83 (± 0.09), 0.68 (± 0.23), and 0.73 (± 0.17), respectively. In Case B, the average peak height ratios in the frontal bone was 0.61 (± 0.23). The DNA concentration was positively correlated with the peak balance, with higher concentrations resulting in a greater peak

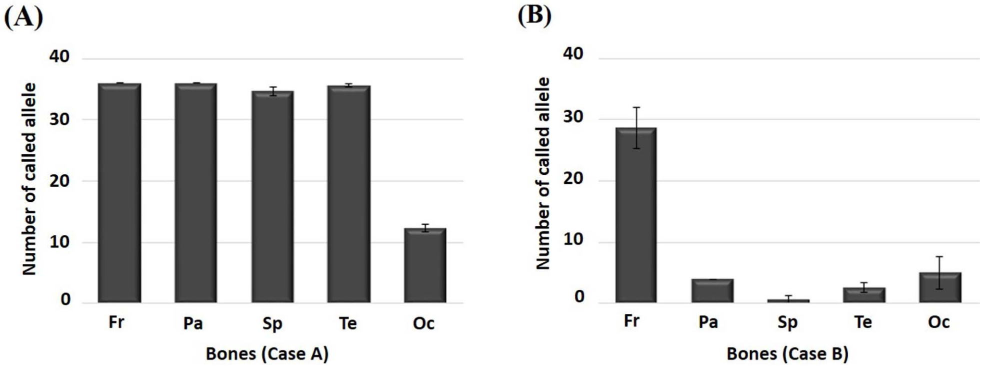

Fig. 4. Number of called alleles in the cranial bones of (A) Case A and (B) Case B. Short tandem repeat (STR) typing was performed by using the GlobalFiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA). Fr, frontal; Pa, parietal; Sp, sphenoid; Te, temporal; Oc, occipital.

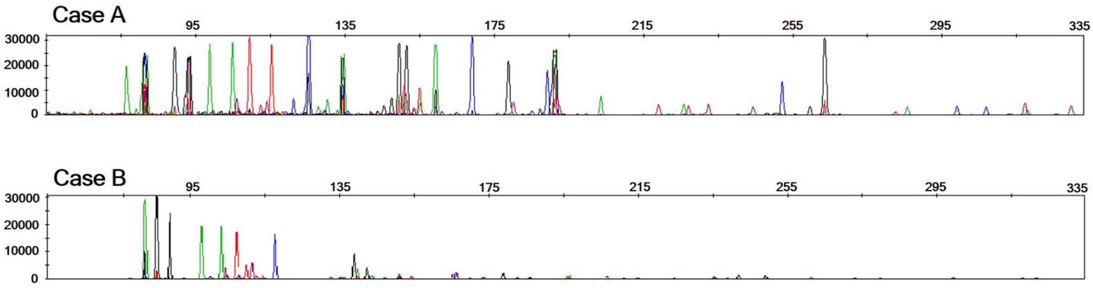

Fig. 5. Representative electropherograms of the frontal bones from Case A and Case B, amplified using the GlobalFiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA). Case B samples showed a more drastic “ski slope” pattern than Case A samples.

balance.

Stochastic effects, which arise randomly due to a low quantity of DNA, can indeed impact the peak balance in STR analysis and its interpretation. When solely considering the count of the called allele in the STR analysis, it might appear that there is minimal variation among samples, as exemplified in cases such as Case A, in which DNA normalization was applied, and the DNA input for PCR was maximized. However, a low quantity of DNA can introduce unpredictable variations in peak balance. By analyzing the peak balance, we were able to observe and subsequently validate this stochastic effect, establishing a clear correlation with the DNA concentration.

3.4. Comparison with previous research

A previous study by Edson *et al.* [12] compared mtDNA yield rates for cranial materials and identified the temporal bone as having the highest value, followed by the frontal bone. In another study by Edson[9], a comparative analysis of DNA testing results based on cranial material and teeth was undertaken using four distinct DNA extraction methods. The findings indicated that the frontal, parietal, and occipital elements were less optimal for DNA extraction than the temporal element.[9] In both these studies, the temporal bone consistently and successfully generated genetic data. The major experimental difference between these two studies and the present study is the temporal bone preparation sites. Edson’s studies[9,12] focused on the petrous portion; however, in the present study, the petrous portion was not considered when sampling temporal bone. The osseous labyrinth, located within the petrous bone, has been demonstrated to contain higher levels of endogenous DNA than any other skeletal element. Nevertheless, in cases involving a complete or reconstructed skull, accessing this labyrinth causes substantial structural damage to either the cranial vault or its base.[11] Therefore, in this study, we primarily sampled easily accessible surfaces to minimize structural damage. Notably, another study investigating the effect of a longer PMI on DNA yield also reported the highest yield in the frontal bone, followed by that in the parietal, temporal, and occipital bones,[3] consistent with our observations for Case A.

Even with the same DNA sample, the DNA yield detection efficiency can vary depending on the analysis methods used. This can be attributed to various factors, including the inhibitor tolerance, amplicon size, and detection target.[3,5,14,15] In Edson's study,[9] DNA samples isolated through organic purification exhibited greater efficiency in mitochondrial Sanger sequencing than those isolated through inorganic purification. However, contrasting results were observed in the STR analysis. Hence, the experimental processes and findings detailed in the present study comprise vital information for forensic DNA laboratories working with bone samples. The differences in the results obtained for the different cases in this study could be attributed to the methodological approaches adopted; nevertheless, the frontal bone consistently exhibited the highest DNA yield among the cranial bones. This can be attributed to factors such as the bone structure, degree of bone decomposition, and presence of inhibitors and microorganisms, which affect DNA yield. [4,16]

Microorganisms are closely related to DNA fragmentation,[17,18] and the porosity and density of the elements could bring about differences in microbial penetration. Cranial bone has a sandwich structure comprising three layers: the outer and inner tables and the middle layer (diploë). Specifically, the outer and inner tables consist of dense cortical bone, whereas the diploë is trabecular (spongy) and has a higher degree of porosity.[19] The structures of the frontal and parietal bones have been compared in terms of the relative thicknesses of the three layers; the outer and inner tables were observed to be thicker for the frontal bone than for the parietal bone.[19] In another study, the average diploic thickness of the occipital bone was found to be greater than that of the frontal bone, regardless of sex.[20] Although the anatomical structures of all five cranial bones were not thoroughly compared, it can be speculated that the cortical thickness and lower porosity of the frontal bone are advantageous for a higher DNA yield.

Comparison of DNA yields between the two cases also suggests that microbial infiltration may have influenced the overall DNA yield. Case B exhibited a lower DNA yield than Case A, indicating a potentially greater level of DNA fragmentation in the cranial bones of Case B. Additionally, electropherograms from STR typing revealed a "ski slope" pattern, indicative of DNA degradation,[8] in Case B (*Fig*. 5). This observation supports the difference in success rates noted between Cases A and B via mtDNA sequencing. While the precise post-mortem interval (PMI) is unknown, Case B, having been buried in the ground, may have been more susceptible to microbial influences from soil-derived microorganisms than Case A.

In general, dense cortical weight-bearing bones and teeth are typically preferred for DNA fingerprinting. However, cranial bones may become the primary focus of forensic analysis when other skeletal elements are unavailable, as observed in Case A and Case B. Despite the representational limitations arising from sampling only two sets of cranial bones for the DNA yield, the cranial elements in our study were obtained from a single individual that had been exposed to the same environment, thus there may have been minimal sampling bias. Thus, we believe that our findings could facilitate the selection of appropriate cranial bones for DNA analysis using minimal samples for forensic identification. Furthermore, adoption of larger sample sizes in future research can enhance the reliability of the results of the present study.

4. Conclusions

In this study, we performed a comparative analysis to evaluate DNA yields from five cranial bones (frontal, parietal, sphenoid, temporal, and occipital bones) from two cases based on three methods, gDNA quantification, mtDNA sequence analysis, and STR typing. For both cases, the frontal bone showed the highest DNA yield. Further, DNA quantification revealed remarkable variations in DNA content among the cranial bones. Our results also showed a correlation between the degree of DNA fragmentation and mtDNA analysis and STR typing results for both cases.

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