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## THE PETROUS BONE: IDEAL SUBSTRATE IN LEGAL MEDICINE?

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# 1 THE PETROUS BONE: IDEAL SUBSTRATE IN LEGAL MEDICINE?

## 2 **Abstract**

3 Over the last few years, palaeogenomic studies of the petrous bone (the densest part of  
4 the temporal bone) have shown that it is a source of DNA in both larger quantities and of better  
5 quality than other bones. This dense bone around the otic capsule has therefore been called the  
6 choice substrate in palaeogenomics. Because the practice of forensic genetics responds to  
7 different imperatives, we implemented a study aimed at (i) understanding how and why the  
8 petrous bone is an advantageous substrate in ancient DNA studies and (ii) establishing whether  
9 it is advantageous in forensic STR typing.

10 We selected 50 individual skeletal remains and extracted DNA from one tooth and one  
11 petrous bone from each. We then amplified 24 STR markers commonly used in forensic  
12 identification and compared the quality of that amplification using the RFU intensities of the  
13 signal as read on the STR profiles. We also performed histological analyses to compare (i) the  
14 microscopic structure of a petrous bone and of a tooth and (ii) the microscopic structure of fresh  
15 petrous bone and of an archaeological or forensic sample.

16 We show that the RFU intensities read on STR profiles are systematically higher in  
17 experiments using DNA extracted from petrous bones rather than teeth. For this reason, we  
18 were more likely to obtain a complete STR profile from petrous bone material, increasing the  
19 chance of identification in a forensic setting. Histological analyses revealed peculiar  
20 microstructural characteristics (tissue organization), unique to the petrous bone, that might  
21 explain the good preservation of DNA in that substrate. Therefore, it appears that despite the  
22 necessity of analysing longer fragments in forensic STR typing compared to NGS  
23 palaeogenomics, the use of petrous bones in forensic genetics could prove valuable, especially  
24 in cases involving infants, toothless individuals or very degraded skeletal remains.

25 **Keywords:** Petrous bones, Teeth, Ancient DNA, Degraded DNA, Histology, STR genotyping

## 26 **Introduction**

27 For over 30 years, human genetics and forensic genetics experts have been concerned  
28 with the amplification of DNA, which may be particularly fragmented, present in low quantities  
29 and /or contaminated [1-3]. The advent of the Polymerase Chain Reaction (PCR) amplification  
30 technique revolutionized the analysis of ancient and/or degraded DNA by allowing in vitro  
31 targeting of DNA fragments and obtaining several million copies [4]. Palaeogenetic studies  
32 subsequently developed, focusing on hard tissues such as bones and teeth, the substrates most  
33 represented in archaeological contexts [5,6]. In the meantime, the discovery of STRs (Short  
34 Tandem Repeats), analysed on automatic sequencers through capillary electrophoresis (CE),  
35 provided a powerful tool for the identification of individuals and the study of close relatives  
36 (kinship, paternity tests) in forensic genetics. Significant, advances have been made in  
37 palaeogenetics (now palaeogenomics) through the development of high-throughput sequencing  
38 systems [7-9]. However, despite the progress made in recent years, selecting a suitable source  
39 of endogenous DNA is still crucial to the success of ancient and/or degraded genetic analyses.  
40 DNA preservation and efficient recovery remain key issues, with taphonomy [10-12] and  
41 environmental conditions being the determinant factors [13].

42 In studies of skeletal remains for genetic identification, investigators often select teeth or  
43 long bones (tibiae, femora) where the compact lamellar layers of bone tissue are relatively dense  
44 allowing for a higher extraction yield than other skeletal elements. These are easily collected  
45 from mass fatality sites or in forensic cases and they represent a microenvironment particularly  
46 favourable to the preservation of the DNA thanks to the adsorption of nucleic acids on the  
47 inorganic bone phase (hydroxyapatite) [14-16], collagen fibers and other fibrous proteins  
48 [17,18]. Among these substrates, teeth appeared to yield the more satisfying results [19-21]. A  
49 study combining genetic and histological analyses demonstrated that cementum was the  
50 material which preserves DNA the longest [22]. Indeed, from a histological standpoint,  
51 cementum and bone present very similar structures [23,24].

52 In recent years however, studies have suggested that the petrous part of the temporal bone  
53 could be even more interesting than teeth as a substrate for DNA extraction [25]. It was  
54 demonstrated that the proportion of endogenous DNA (as a percentage of total DNA extracted)  
55 obtained from petrous bone is 4 to 16 times larger than the proportion obtained from dental  
56 elements and up to 183 times superior than the proportion obtained from other skeletal elements  
57 (ribs, metacarpal or metatarsal bones) [26]. Since then, it has been specified that it is the densest

58 part of the otic capsule or osseous labyrinth, corresponding to the cochlea, that allows scientists  
59 to obtain greater quantities of endogenous DNA [27]. Several studies confirmed the status of  
60 the petrous bone as the substrate of choice for analyses of ancient and/or degraded DNA [28,29].

61 Conventional approaches used in genetic identification, usually STR-based, are not  
62 highly sensitive to the presence of exogenous DNA. They do however require the amplification  
63 of long fragments (between 70 and 450 bp), whereas Next Generation Sequencing (NGS)  
64 techniques, also called Massively Parallel Sequencing (MPS), allow sequencing from very  
65 small fragments (< 50bp), but are highly sensitive to the presence of exogenous DNA since  
66 they do not discriminate between DNA from different sources. A study using STR analysis on  
67 archaeological samples has already suggested that DNA extracted from petrous bones is also  
68 more likely to yield complete STR profiles than DNA extracted from other substrates [30].

69 We endeavoured to replicate those results, apply the same comparison to forensic samples  
70 and identify the specific circumstances in which the use of a petrous bone might be  
71 advantageous. Given that both petrous bones and teeth have already been shown to yield better  
72 results than long bones [30], our study includes only the two first sample types. We also  
73 performed histological analyses to determine and illustrate the peculiar microscopic structure  
74 of the petrous bone, including the otic capsule, which has been described as a protection against  
75 pathogen intrusion [31].

76 Using 50 pairs of samples, each a petrous bone and a tooth from one individual exhumed  
77 in an archaeological or forensic context, we compared the RFU intensities of the amplification  
78 profiles of STR markers commonly used in forensic genetics and the number of amplified  
79 alleles. We also compared two archaeological series of more than 20 samples that had  
80 undergone very different environmental conditions since inhumation, in order to evaluate the  
81 effect of post-mortem DNA degradation on RFU intensities and the number of amplified alleles.  
82 Histological comparisons of a fresh tooth and a fresh petrous bone, as well two petrous bones  
83 collected from an archaeological and a forensic context, allow us to better identify the limits of  
84 the advantages presented by the petrous bone as a substrate for the extraction of degraded DNA.

## 85 **Materials and methods**

### 86 **Samples used for genetic analyses**

87 Genetic investigations were carried out on 65 petrous bones and 50 teeth (a total of 115  
88 samples). Among the 65 petrous bones, 55 were from archaeological excavation sites and 10  
89 were from forensic cases. Among the 50 teeth, 40 were from archaeological excavations and  
90 10 were from the same forensic cases stored away from light at room temperature for more than  
91 20 years [Table 1]. The six archaeological sites were: two sites in north-eastern France (4<sup>th</sup>-2<sup>nd</sup>  
92 century B.C.E. and 5th century C.E., respectively), a Hungarian site (9<sup>th</sup> century C.E.) and three  
93 Mongolian sites (5<sup>th</sup>-3<sup>rd</sup> century B.C.E., 3<sup>rd</sup>-1<sup>st</sup> century B.C.E. and 1<sup>st</sup> century B.C.E. to 1<sup>st</sup>  
94 century C.E.).

### 95 ***Sample processing***

96 Multirooted teeth (molars and premolars) were favoured because of a higher root area and  
97 a larger pulp volume. When selecting teeth, closed apices were preferred. Because lesions  
98 (cracks, cavities, etc.) promote the entry of bacteria (and therefore contamination of the DNA  
99 sample) the damaged teeth were discarded. During the decontamination phase, the teeth were  
100 meticulously cleaned with a sterile compress soaked in a diluted sodium hypochlorite solution,  
101 rinsed with sterile Nuclease-Free water (Euromedex, cat. n°UW0900-A) and then dried with a  
102 sterile compress. Each face was then exposed to short-wavelength UV for 30 minutes. Entire  
103 teeth were then reduced to powder by cryogenic grinding (liquid nitrogen cryocrusher 6870  
104 freezer/Mill). For each tooth, approximately 250 mg of powder were then transferred into a 1.5  
105 ml microtube, taking care to separate the large particles (corresponding to the enamel) from the  
106 finer particles (corresponding to the other structures of the tooth). The equipment needed for  
107 cryogenic grinding (cryogenic tubes, caps and firing pins) had previously been washed with  
108 bleach, rinsed with deionized water (Euromedex, cat. n°22800-05), dried with ethanol 70%,  
109 and then exposed to a Bio-Link 254 nm irradiator (Crosslinker).

110 Some of the petrous bones were already isolated, having separated from the skull before  
111 or during excavation. Those who were attached to other cranial bones were separated manually.  
112 Extraction was carried out using a diamond saw mounted on a Dremel<sup>®</sup> under an extraction  
113 hood. To remove contaminants before any drilling operation, the surface of the petrous bone  
114 was mechanically abraded 1 mm deep using a Dremel<sup>®</sup> mounted cutter. The drilling was then  
115 carried out using a ball cutter orientated towards the cochlea, the densest part of the otic capsule.

116 The resulting fine powder was collected in a sterile cup. This original protocol/treatment of the  
117 petrous bone performed within our laboratory allows us to properly decontaminate the petrous  
118 part and yet return a near-intact petrous bone to archaeologists and anthropologists or judicial  
119 authorities. This method differs from that of Sirak and his collaborators [32], that aims to obtain  
120 bone powder without detaching the temporal bone from the skull. In the present case, the careful  
121 and precise cut allows repositioning of the petrous part once the powders have been collected.

### 122 *DNA extraction and amplification*

123 From bone and tooth powders (approximately 250 mg), DNA extraction was performed  
124 during an all-night incubation at 50°C with an extraction buffer consisting of EDTA (0,5M),  
125 DTT (1M) and proteinase K (20mg/ml). To increase the efficiency of DNA extraction a  
126 decalcification step was performed (incubation overnight with EDTA 0,5M). The extracted  
127 DNA was then purified on silica columns and then concentrated [33]. Two independent DNA  
128 extractions were completed from each sample. STR analysis was performed using a commercial  
129 human genetic identification kit, the GlobalFiler<sup>®</sup> PCR (Thermo Fisher Scientific)  
130 amplification kit, following the supplier's recommendations. It allows the simultaneous  
131 analysis of 24 genetic markers, including 21 autosomal markers (D3S1358, vWA, D16S539,  
132 CSF1PO, TPOX, D8S1179, D21S11, D18S51, D2S441, D19S433, TH01, FGA, D22S1045,  
133 D5S818, D13S317, D7S820, SE33, D10S1248, D1S1656, D12S391, D2S13), two Y  
134 chromosome markers (DYS391, Y indel) and a sex determination marker (amelogenin gene).  
135 The sizes of the fragments amplified using this kit range from 70 to 450bp. PCR amplification  
136 reactions were performed using a thermocycler (Biometra). We followed the manufacturers'  
137 protocols, except for the number of PCR cycles, which was increased from 29 to 32 for the  
138 archaeological sample. The amplified products were then analysed using an automatic 3500  
139 Genetic Analyzer (Applied Biosystems) using polymer POP7. The DNA profiles were analysed  
140 using GeneMapper<sup>®</sup>v4.1 software. The analytical threshold for allelic calls was set at 50 RFU,  
141 in accordance with our internal validation guidelines. At least two independent extractions  
142 underwent amplification for each sample and consensus DNA profiles were established by  
143 retaining the alleles that had been typed at least twice for each sample [**Online Materials Table**  
144 **S1**].

### 145 *Precautions taken to avoid contamination*



146 This work was carried out in rooms dedicated to the study of degraded DNA. The pre-  
147 PCR and post PCR laboratories are located on different floors. The pre-PCR laboratory was  
148 strictly dedicated to ancient DNA, with positive pressure and UV light irradiations. Very strict  
149 rules were followed, between each manipulation; benches and supplies were cleaned with  
150 bleach, ultrapure water and DNA away (Dominique Dutscher, cat. n°038188) and placed under  
151 UV light. The manipulators wore appropriate equipment: overshoes, a facial mask, a mobcap,  
152 a lab coat and gloves. The genetic profile of all the people in contact with the samples was  
153 established and compared with the DNA profiles of ancient specimens. For each sample (tooth  
154 or petrous bone), multiple DNA extractions and PCR amplifications were performed and  
155 negative controls were included in each experiment (one extraction or amplification blank for  
156 every four samples).

### 157 *Statistical Analysis*

158 The quality of DNA amplification was measured for each substrate using the peak RFU  
159 (Relative Fluorescence Units) intensities of the amplified alleles for all 24 markers [**Online**  
160 **Materials Table S2**]. For the following analyses, we retained the results of only one multiplex  
161 amplification for each sample, the most successful (that is to say the amplification yielding the  
162 most allele calls and, when comparing profiles with identical numbers of allele calls, the highest  
163 RFU intensities).

164 Alleles were ranked by size from shortest to longest into 5 intervals: <120bp, 120-180bp,  
165 180-240bp, 240-300bp and >300bp and the RFU intensity of homozygotes (only one peak) was  
166 divided by two [**Online Materials Table S3**]. Some markers encompass alleles belonging to  
167 more than one interval.

168 For all 100 paired samples (with one tooth and one petrous bone from 50 individuals), the  
169 number of markers successfully amplified for each STR profile was also compared. Markers  
170 were divided into two categories: “short”, if all alleles were less than or equal to 240bp in length  
171 and “long”, if all alleles were more than 240bp in length. This 240bp demarcation was chosen  
172 to exclude as few markers as possible. For this test, typed alleles of the marker D21S391 fell  
173 into both categories and were thus distributed in one or the other [**Online Materials Table**  
174 **S4a**].

175 We tested the difference in mean RFU intensities between size intervals using Student  
176 t-tests. Differences were considered significant for p-values <0.05. We tested the difference in  
177 the number of short or long markers successfully amplified from tooth or petrous bone using a

178 paired t-test with the same significance threshold. Statistical tests were performed using in-  
179 house R scripts [34].

## 180 **Samples used for histological analyses**

181 Histological sections were carried out on (i) a fresh tooth and a fresh petrous bone in order  
182 to compare the microscopic structure of these two organs and (ii) two undated archaeological  
183 petrous bones in order to analyse microscopic tissue degradation within these structures.

### 184 *The fresh dental sample*

185 The human dental sample was collected from a patient undergoing a conventional wisdom  
186 tooth extraction. It was therefore a left superior third molar (tooth N28). The freshly extracted  
187 tooth was immediately fixed in 10% buffered formalin (pH = 7,2) for 6 weeks to stabilise tissue  
188 structures. At the end of the fixation, the tooth was sagittally halved in two segments by means  
189 of a diamond saw mounted to a Dremel<sup>®</sup> to expose the pulp and root canal. Both dental  
190 fragments were then decalcified in 10% EDTA (pH 7.2). Decalcification time was 27 days.  
191 After completion of the decalcification, both fragments were dehydrated using increasing  
192 graded series of ethyl alcohol, cleared in xylene and impregnated with melted paraffin, in a  
193 vacuum infiltrating processor (VIP Tissue-Tek 6, Sakura<sup>®</sup>). Each dental sample was embedded  
194 separately in one block of paraffin wax by means of an embedding station (Tissue Tek III of  
195 Sakura<sup>®</sup>). Paraffin with a high melting point of 62-64°C (Ref: 19304-01, EMS, Philadelphia)  
196 was retained to provide a more solid matrix to the tooth samples and consequently easier  
197 sectioning. Serial sections of 5 µm thickness were cut with a motorized microtome (Jung  
198 Autocut, Leica<sup>®</sup>) and stained with (i) hematoxylin and eosin (H&E) to assess the general  
199 morphology and (ii) Picrosirius (PS) using Sirius red F3B [C.I. 32782] as a dye for the study of  
200 collagen network viewed under polarised light [35-37]. All stained slides were examined with  
201 a light and polarised microscope (Axiophot, Zeiss<sup>®</sup>) connected to an AxioCam MRc5 camera  
202 (Zeiss<sup>®</sup>). Sections were also scanned using the PathScan<sup>®</sup> Touch software (Excilone) connected  
203 to an Axiophot microscope (Zeiss<sup>®</sup>) with a CMOS camera.

### 204 *The fresh petrous bone sample*

205 The human temporal bone sample was taken from body donated to science, through our  
206 partnership with the laboratory of anatomy of the Faculty of Medicine of Strasbourg. Once the

207 petrous part of the temporal bone was extracted and carefully cleaned by a medical examiner,  
208 it was fixed in 10% buffered formalin (pH = 7,2) for 6 weeks. At the end of the fixation, the  
209 isolated petrous bone was cut according to a sagittal plane using a diamond saw mounted on a  
210 Dremel<sup>®</sup> to expose the otic capsule. The two fragments exposing the three parts of the petrous  
211 bone (cochlea, vestibule and semi-circular canals) were decalcified in Kristensen's solution  
212 (formic acid and sodium formate). This solution was prepared in the laboratory according to  
213 the procedure described in the literature [38]. This method of decalcification uses a weak acid  
214 and is therefore less damaging. Decalcification time was 18 days. After completion of the  
215 decalcification, the following histoprocessing (dehydration, embedding, cutting and staining)  
216 was similar to the one used to process the tooth. One additional staining was performed, i.e.  
217 Safranin O / Fast green (SO/FG) to highlight cartilage [39].

### 218 *The two undated archaeological petrous bones samples*

219 The first petrous bone came from a temporal bone that was already detached from the  
220 skull and stored in a dark room at room temperature between 10 to 15°C for more than 20 years.  
221 The second petrous bone was extracted using a diamond saw mounted on a Dremel<sup>®</sup> from a  
222 more recent skull that had been stored at room temperature for about 10 years. Both were  
223 undated archaeological skeletal remains and were stored in a dry cardboard box at room  
224 temperature between 10°C and 15°C. These two petrous bones were fixed in 10% buffered  
225 formalin (PH=7.2), for 6 weeks and 8 weeks, respectively. They were subsequently decalcified  
226 in 10% EDTA (pH 7.2). Decalcification time was 30 days for both samples. After completion  
227 of the decalcification, the following histoprocessing (dehydration, embedding, cutting and  
228 staining) was similar to the one used to process the fresh petrous bone.

## 229 **Results**

### 230 *Amplification is more reliable using DNA extracted from petrous bones*

231 For the three first allele size intervals (up to 240bp in length, “short” fragments), the  
232 amplification of DNA from petrous bones consistently yields higher mean RFU intensities than  
233 the amplification of DNA from teeth (p-values: 5.142e-07, 3.313e-06 and 1.51e-04) [**Figure 1;**  
234 **Table 2**]. This is not the case for longer fragments (over 240bp), for which mean RFU intensity  
235 does not significantly differ between DNA extracted from teeth and DNA extracted from  
236 petrous bones. This result stands when comparing only archaeological teeth to archaeological  
237 petrous bones, or only forensic teeth to forensic petrous bones [**Table 2**].

### 238 *The degradation of DNA in petrous bones is subject to environmental factors*

239 We compared 24 petrous bones from one Mongolian necropolis-Saghil (5<sup>th</sup>-3<sup>rd</sup> century  
240 B.C.E.) and 22 from one Hungarian necropolis (9<sup>th</sup> century C.E.). The first is situated in the  
241 Altai region of Mongolia which is generally cold and extremely arid, with mild summers. The  
242 second necropolis is situated in more temperate Hungary, which is significantly warmer and  
243 more humid.

244 We show that samples from the first site consistently yield better quality DNA for all five  
245 size intervals [**Table 2**], although, in both cases, longer fragments are amplified at lower RFU  
246 intensities than shorter fragments. This highlights the discrepancies in preservation between  
247 different archaeological sites, that are not necessarily linked to the age of the samples since, in  
248 this case, the older samples yielded better quality DNA. Rather it is environmental conditions  
249 that have the greater influence over preservation, as previously described and expected in such  
250 samples [40,41].

### 251 *More complete profiles are obtained using DNA from petrous bones*

252 Paired samples (one petrous bone, one tooth) from 50 individuals were used to compare  
253 the effectiveness of DNA extracted from both substrates in delivering complete autosomal STR  
254 profiles. Comparing numbers of amplified STR loci, we show that DNA extracted from petrous  
255 bones more often yields “successful allelic calls” (i.e. expected heterozygous alleles are  
256 observed) for markers relying on long fragments (over 240bp, p-value: 0.02473) and for

257 markers relying on shorter fragments (< 240bp, p-value: 0.03974) [**Online Materials Table**  
258 **S4b**]. This results in more complete STR profiles when using petrous bones, especially when  
259 teeth do not permit the amplification of longer fragments. Based on the multiple amplifications  
260 from independent DNA extracts of the same individual, we did not observe any discrepancy in  
261 allelic calls between substrates, although some heterozygotes could be mistaken for  
262 homozygotes when one allele had not been amplified using DNA extracted from teeth.

### 263 *Histology of DNA preservation in teeth*

264 Most of the volume of the tooth is made up of dentine, as shown on the sagittal section  
265 of the left superior third molar [**Figure 2A**]. Dentine is mostly composed of type I collagen  
266 [**Figure 2B**] and observation in polarized light shows that these collagen fibers are grossly  
267 parallel [**Figure 2B1**]. Cementum (an entirely mineralised matrix) is also constituted of type I  
268 collagen fibers, oriented parallelly to the axis of the root [**Figure 2B2**]. Neither dentine nor  
269 acellular cementum are vascularised or innervated. Nucleated cells are mainly visible in root or  
270 crown pulp. There is a high concentration of odontoblasts at the pulp-dentine junction [**Figure**  
271 **2A1**] and cementoblasts can also be observed in cellular cementum [**Figure 2A2**].

### 272 *Histology of DNA preservation in petrous bones*

273 Distinct histological structures within the petrous bone are highlighted on a sagittal  
274 section [**Figure 3A**]. These structures are especially visible around the cochlea [**Figure 3B**],  
275 where a protective shell divides two regions [**Online Materials Figure S1**]. The region  
276 bordering the cavities of the inner ear (cochlea and semi-circular canals) is a type I collagen  
277 matrix, rich in nucleated cells, inside which under-coloured areas can be observed. Safranin-  
278 O/Fast green (SO/FG) indicates that these areas are made up of cartilaginous tissue. Observation  
279 under polarised light shows the different organisation of collagen fibers: unorganised in the  
280 border region of the inner ear and parallel inside the protective shell segregating that region  
281 from the rest of the petrous bone. The unorganised border region is also devoid of secondary  
282 osteons, which suggests the absence of vascularisation [**Online Materials Figure S2**].

283 *The impact of environmental conditions on petrous bone microstructure*

284 We performed histological analyses on a “degraded” petrous bone and a “very  
285 degraded” petrous bone, in order to observe the state of the structures previously described after  
286 degradation. The sagittal section of the “degraded” bone [**Figure 4, Online Materials Figure**  
287 **S3**] shows the same organisation, with a protective shell surrounding the border region of the  
288 inner ear. Because of autolysis, cell nuclei cannot be observed in this degraded sample [**Online**  
289 **Materials Figure S4**].

290 The sagittal section of the “very degraded” sample [**Online Materials Figure S5**] shows  
291 the same signs of degradation (absence of cell nuclei) inside the border region of the inner ear  
292 [**Online Materials Figure S6**], and further signs outside of that region. Although the presence  
293 of the protective shell and the orientation of collagen fibers are unchanged [**Online Materials**  
294 **Figure S7**], bone tissue outside the protective shell has undergone significant bioerosion.

## 295 **Discussion**

### 296 *The protective shell around the otic capsule preserves it from degradation*

297 The comparison of histological structures in a degraded petrous bone and a very  
298 degraded petrous bone showed that, although there is significant bioerosion around the otic  
299 capsule, histologically distinct regions can still be observed: a protective shell segregates an  
300 area of hyper-mineralised bone tissue where collagen fibers are unorganised [42]. Safranine-  
301 O/Fast green also highlighted the presence of glycoproteins (constituents of the cartilaginous  
302 matrix) within this protected area (the border region of the inner ear), even in very degraded  
303 petrous bone. Since the degradation of glycoproteins is normally a rapid process, their  
304 persistence at this stage is another indication of the isolation of the otic capsule from other  
305 skeletal elements [Online Materials Figure S7].

### 306 *The conditions of degradation in petrous bones vary between archaeological sites*

307 Physical, biological and chemical alterations of skeletal remains are the determining  
308 factors in the long-term preservation of DNA [10,12]. Cold and dry environments are the most  
309 favourable as previously shown [40,41].

310 The comparison of STR amplification results in a 9<sup>th</sup> century C.E. Hungarian necropolis  
311 (temperate climate) and a 5<sup>th</sup>-3<sup>rd</sup> century B.C.E. Mongolian necropolis-Saghil (cold and dry  
312 climate) confirmed this issue. Across all allelic size intervals, petrous bone material from the  
313 second site yields higher mean RFU intensities. This demonstrates that whatever advantage  
314 exists in preferring petrous bones to teeth as primary substrates for DNA amplification, there  
315 are important discrepancies between different petrous bones. Some samples might be too  
316 degraded for reliable analysis. It should be noted however, that teeth undergo the same  
317 degradation process under the same conditions.

### 318 *The absence of vascularisation prevents exogenous contamination*

319 As shown on the sagittal section of the petrous bone, the border region of the cochlea  
320 and the semi-circular canals contains chondrocyte residue. These components are typical of  
321 immature bone. Moreover, secondary osteons cannot be observed in that region. Since  
322 secondary osteons are centred around blood vessels, innervated and intervene in the

323 development and remodelling of bone tissue, this observation is consistent with limited  
324 vascularisation and the absence of remodelling, as observed in previous studies [27]. Without  
325 blood supplied to this region, the otic capsule is preserved from the introduction of some  
326 microorganisms, while dental pulp is densely vascularised and has been shown to contain  
327 greater microbial diversity [31]. The border region of the inner ear is therefore relatively  
328 protected from exogenous contamination, both because of its anatomical isolation and its  
329 particular histological structure.

### 330 *Palaeogenomic methods rely on short endogenous fragments*

331 In NGS approaches used in palaeogenomics, DNA libraries are created using all the  
332 DNA available in a sample, human or otherwise. DNA is recovered from microorganisms that  
333 occupied the sample before death, colonised it after death and/or participated in its degradation.  
334 Although this exogenous DNA is eliminated by the NGS methodology, the selection of suitable  
335 samples influences the quantity of DNA recovered.

336 In palaeogenomics, substrates containing a high proportion of endogenous DNA are preferred  
337 in order to obtain genomes with sufficient coverage. The endogenous/ exogenous ratio is  
338 therefore a decisive factor. For these reasons, the petrous bone is a choice substrate for  
339 palaeogenomics: (i) its rapid formation *in utero* and isolation [43] favour the good preservation  
340 of DNA; (ii) this anatomical isolation also protects the otic capsule from exogenous  
341 contamination (more vascularised bone is more susceptible to the introduction of  
342 microorganisms); (iii) the petrous bone is not remodelled during life and presents a high  
343 concentration of osteocytes [44]; (iv) large quantities of DNA, even fragmented, are adapted to  
344 NGS sequencing techniques, that focus on short fragments. On the contrary, PCR-based  
345 techniques generally rely on fragments longer than 70 bp [45]. Although this technique is  
346 equally reliable, in the case of highly degraded and fragmented DNA it becomes less effective.

### 347 *Forensic genetics rely on the recovery of complete STR profiles*

348 The endogenous DNA yield of the otic capsule is not the main benefit of using petrous  
349 bones in forensic investigations. Exogenous contamination is in fact less of an issue when  
350 amplifying human-specific markers that cannot be found in microbial DNA sequences.  
351 Contamination by exogenous human DNA (during analysis or handling) is a more pressing  
352 issue.



353 Forensic analysis still relies mainly on STR genotyping, although other markers (e.g.  
354 SNP, mitochondrial sequences) do have specific applications. Because the usefulness of  
355 databases resides in comparing an STR profile with reference samples, crime-scene samples or  
356 samples from unidentified corpses, more complete STR profiles are more likely to lead to  
357 successful forensic investigations [46].

358 Our results indicate that more complete profiles (more loci amplified) are obtained using  
359 DNA extracted from petrous bone than DNA extracted from teeth. We showed that this is due  
360 to the better amplification of longer fragments in the first substrate. Complete profiles can  
361 however be obtained using teeth when they are well-preserved. This implies that, in very  
362 degraded skeletons, selecting petrous bones for DNA extraction could be a simple way to  
363 increase the probability of obtaining complete STR profiles and limit allelic drop-out.

## 364 **Conclusion**

365 Histological investigations have shown the isolation of the otic capsule favours its  
366 preservation from exogenous contamination. Its atypical structure, with immature bone that is  
367 not remodelled and less vascularised than tooth pulp, also contributes to maintaining a high  
368 proportion of endogenous DNA. The protective shell itself limits bioerosion and the physical  
369 degradation of the tissue around the inner ear.

370 Genetic analyses have shown that DNA extracted from the petrous bones yields better  
371 results than DNA extracted from teeth: (i) short fragments are amplified at higher RFU  
372 intensities and (ii) long fragments are more likely to be amplified. The first result (along with  
373 the absence of exogenous contaminants) made the petrous bones the preferred sample for  
374 palaeogenomic studies. The second result implies that it yields complete STR profiles more  
375 often than teeth. This is an indication that petrous bones could also be the ideal substrate for  
376 forensic genetics investigations.

377 Although the use of the petrous bone should not be systematic (teeth can also provide  
378 complete STR profiles), it could be especially advantageous when studying very degraded  
379 skeletons, for example after carbonisation of the corpses or in particularly deleterious  
380 taphonomic conditions, or for toothless individuals. Finally, as forensic genetics incorporate  
381 Next Generation Sequencing (NGS) approaches, using petrous bones could become as  
382 beneficial as it has been to palaeogenomics, for the same reasons.

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## Legend of figures

### Figure 1: Distribution of the average RFU intensities for the 50 petrous bone/teeth pairs

\*\* p-value inferior to 0.05 for a student t-test: mean RFU intensity for DNA extracted from petrous bones is significantly superior to mean RFU intensity for DNA extracted from teeth.

### Figure 2: Histomorphological analysis of a freshly extracted tooth

Top, presentation of a section of human mandibular molar and its support structures. *According to Franck H. Netter, Atlas of Human Anatomy, 5th edition, 2011, p57.*

**A.** Histological section of a superior third molar cut according to a sagittal plane. **A1.** A higher magnification of the pulp-dentine junction shows blue points corresponding to the nuclei of the odontoblasts. **A2.** A higher magnification at the cementum level shows blue points (*black arrows*) corresponding to the nuclei of the cementoblasts. **B.** The collagen fibers are stained red. **B1.** Observed under polarized light the collagen fibers are grossly parallel and run at a right angle to the long axis of the root. **B2.** At the cementum level, the collagen fibers are thinner and run parallel to the long axis of the root.

### Figure 3: Histomorphological analysis of a freshly petrous bone sample

Top left, sagittal section of the fresh petrous bone. Top right, shape and situation of the otic capsule, *according to Legent F, Perlemuter L, Vandenbrouck C. Anatomy notebooks O. R. L., 2nd edition.1968 -1975 Masson et Compagnie, Editeurs, Paris.*

**A.** Histological section including the otic capsule (*square*) and a peculiar bone structure (*asterisk*).

**B.** The peculiar structure stained with picrosirius (PS) shows under-colored areas (*white arrows*). The safranin-O/Fast green demonstrates the presence of cartilage at the level of under-colored areas (*white arrows*). Higher magnification of the same structure with Hematoxylin and Eosin (H&E) shows the presence of many blue colored nuclei (*black arrows*). The picrosirius stained area observed under polarized light (PS + Polarized light) demonstrated the presence of collagen fibers with no particular orientation whereas the fibers constituting the protective shell are grossly parallel (*bracket*).

### Figure 4: Histology section through the cochlea

**A.** Hypermineralized bone with interlaced collagen bundles with no predominant orientation (*star*) and absence of bone remodelling enclosed in a thick layer of lamellar bone with collagen fibers running parallel (*bracket*).

**B.** Higher magnification Staining: Picrosirius + polarization

Collagen fibers constituting the protective shell are grossly parallel (*bracket*) whereas other fibers have no particular orientation (*star*).

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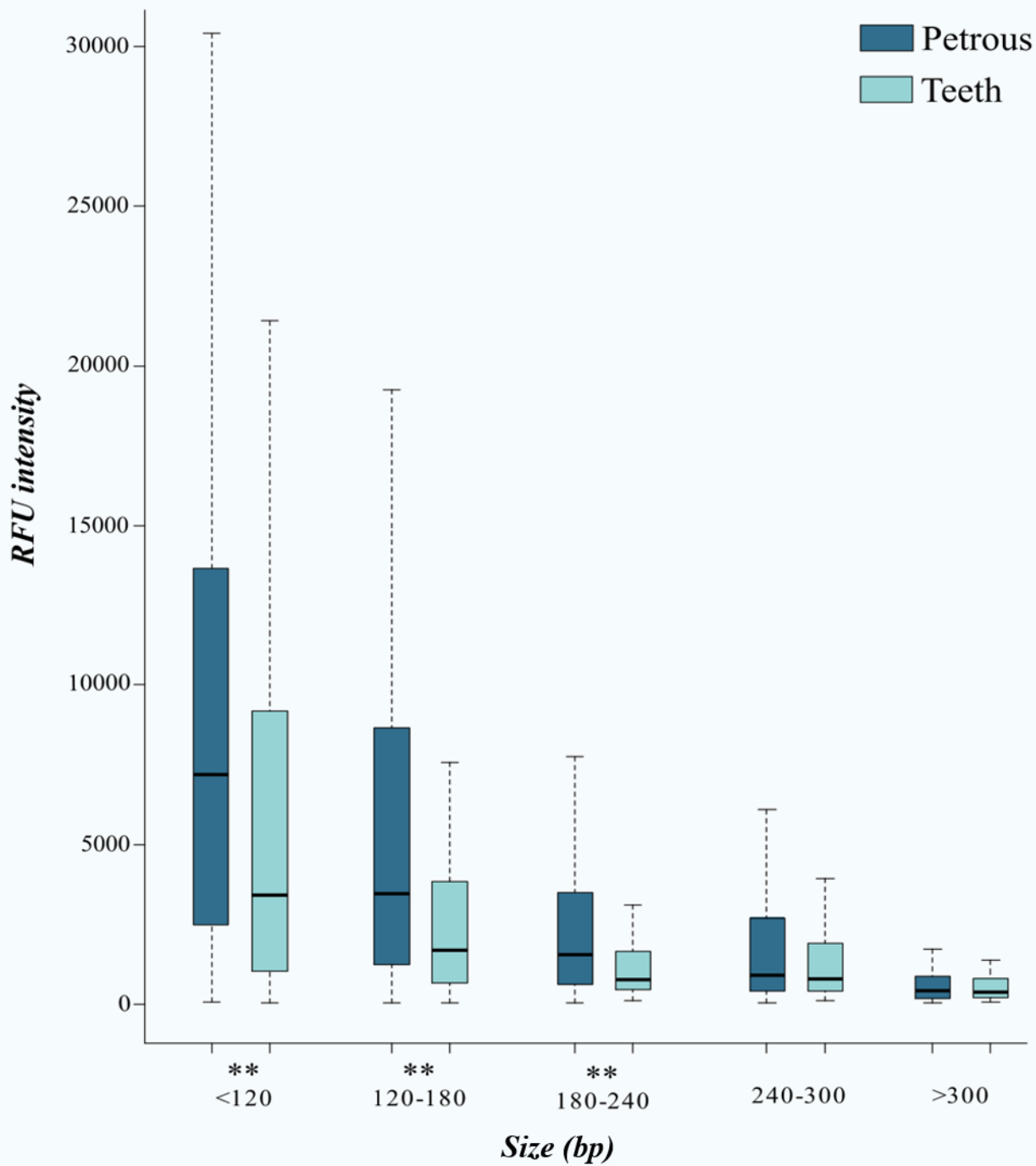
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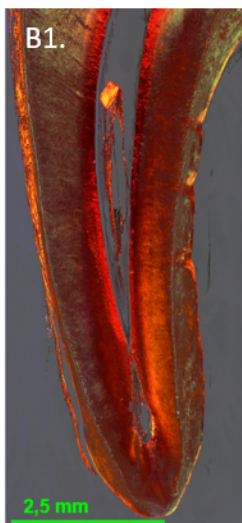
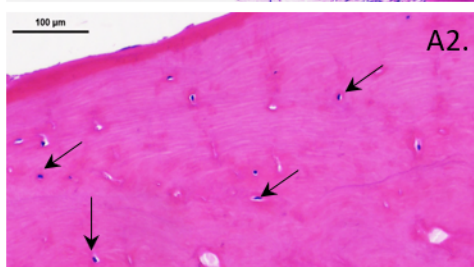
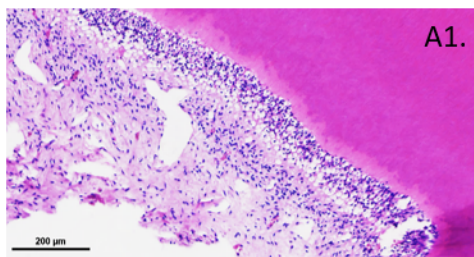
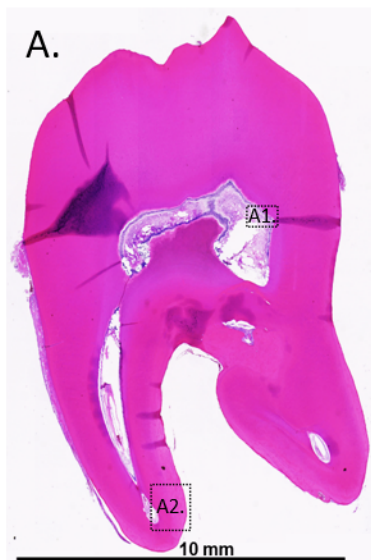
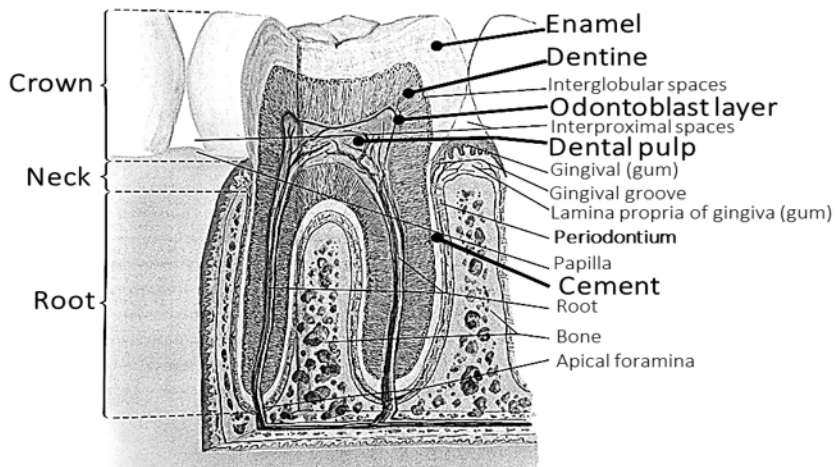
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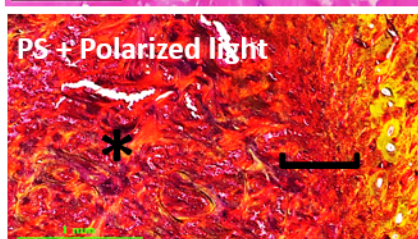
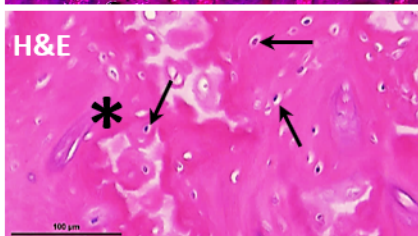
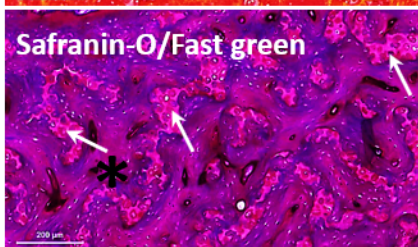
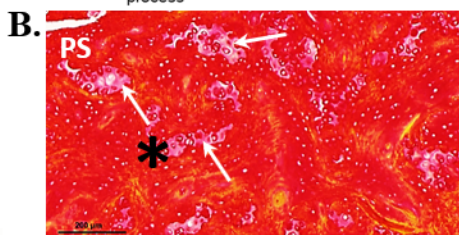
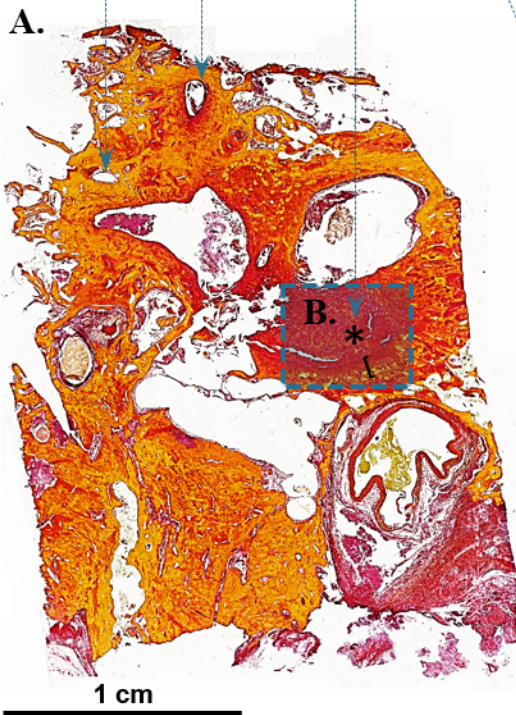
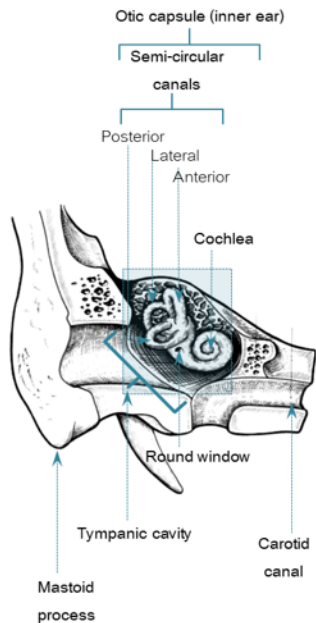
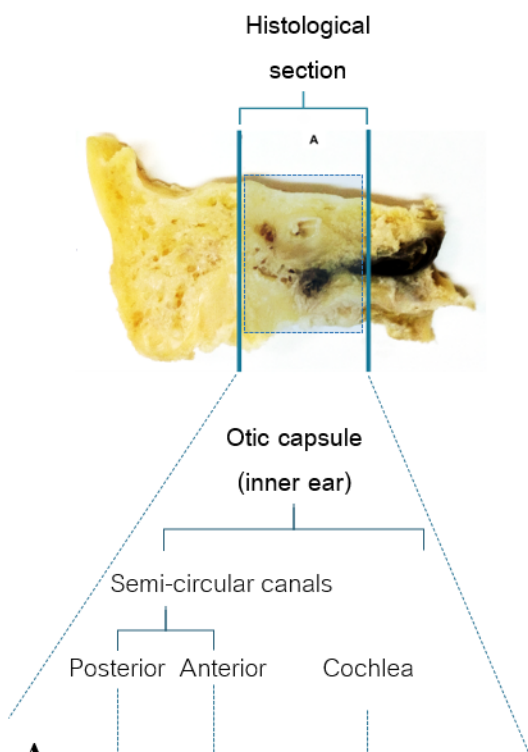


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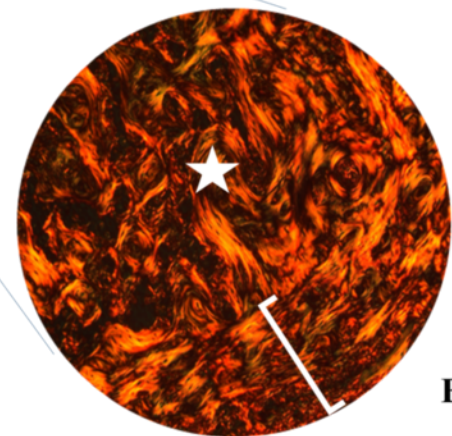
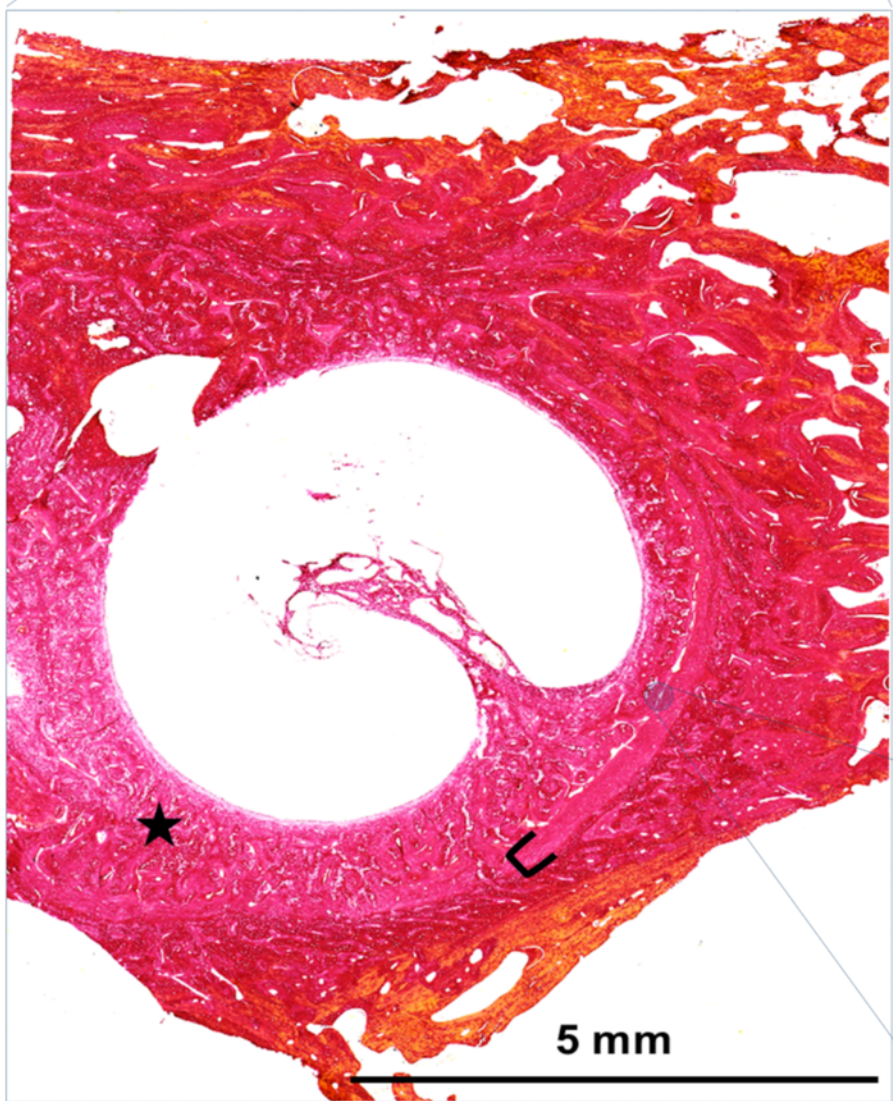
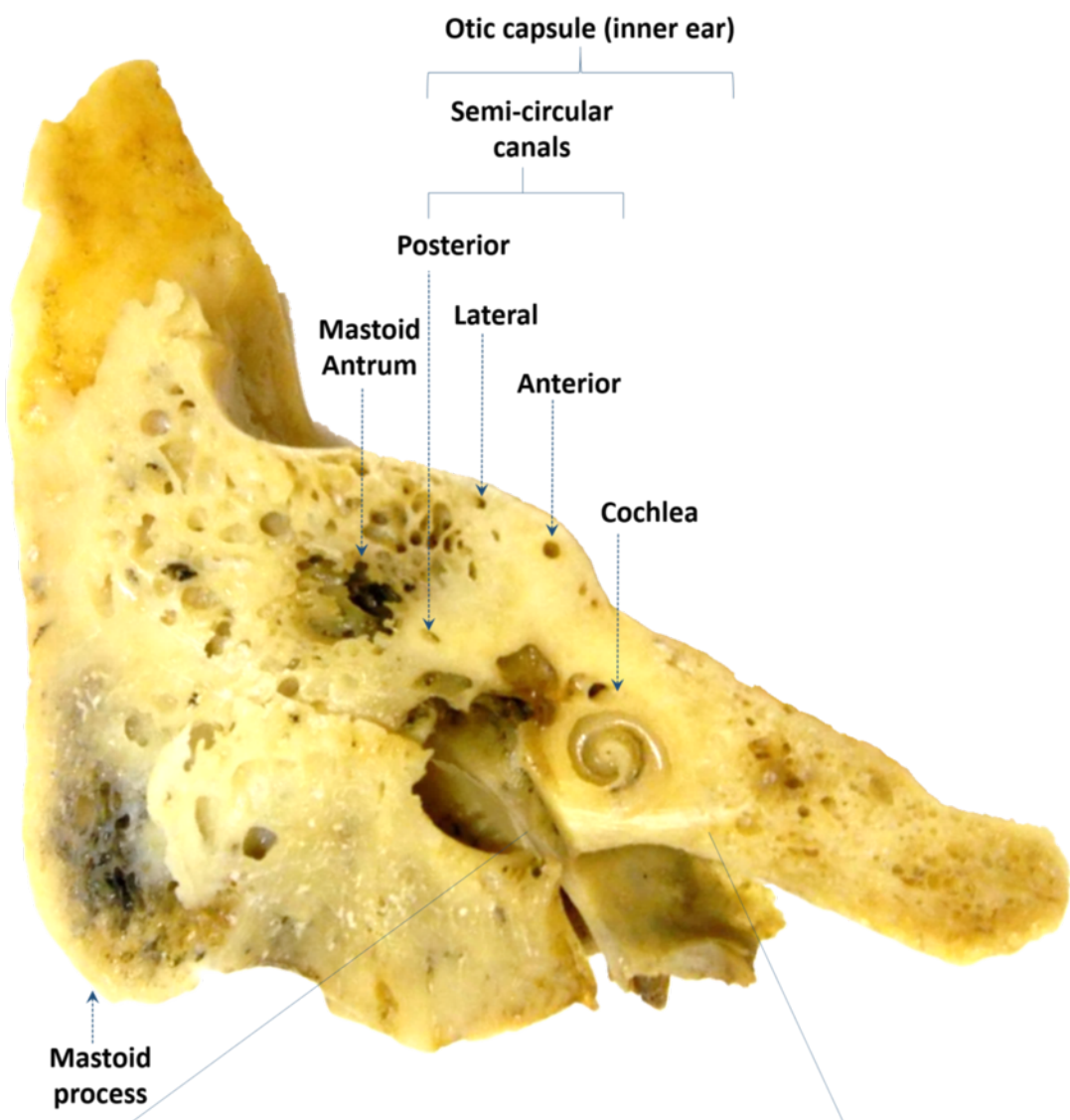












**A.** Histology section through the cochlea  
Staining: Picrosirius

**B.** Higher magnification Staining:  
Picrosirius + polarization

<b>Samples</b>	<b>Geographical origine/Site</b>	<b>Datation</b>	<b>N</b>	<b>Substrates</b>
<b>S1-S24</b>	Altai Mogol/Saghil	5 <sup>th</sup> -3 <sup>rd</sup> century B.C.E.	24	9 pairs tooth/petrous bone and 13 isolated petrous bones
<b>M1-M2</b>	France/Marsal	4 <sup>st</sup> -2 <sup>nd</sup> century B.C.E.	2	2 pairs tooth /petrous bone
<b>B1-B4</b>	Mongolia/Burgast	3 <sup>rd</sup> -1 <sup>st</sup> century B.C.E.	4	4 pairs tooth /petrous bone
<b>T</b>	Mongolia/Xiongnu	1 <sup>st</sup> century B.C.E – 1 <sup>st</sup> century C.E.	1	1 pair tooth /petrous bone
<b>N1-N2</b>	France/Niedernai	5 <sup>th</sup> century C.E.	2	2 pairs tooth /petrous bone
<b>H1-H22</b>	Hungary	9 <sup>th</sup> century C.E.	22	22 pairs tooth /petrous bone
<b>X1-X10</b>	-	Present day (forensic)	10	10 pairs tooth /petrous bone
			<b>65</b>	<b>50 pairs tooth /petrous bone</b>

**Table 1: Sample overview**

**N:** number of genotyped individual. A total of 65 skeletons were sampled in this study. We have extracted DNA from one tooth and one petrous bone from all individual skeletal remains except for the Saghil site where we could only process 9 teeth and 9 petrous bone.

	< 120bp	120-180bp	180-240bp	240-300bp	> 300bp
All petrous bones/teeth	5.142e-07*	3.313e-06*	0.000151*	0.09526	0.1671
Archeological petrous bones/teeth	0.001464*	0.001933*	0.003069*	0.3351	0.1672
Forensic petrous bones/teeth	2.018e-07*	2.175e-05*	0.008736*	0.03866*	0.7509
Petrous bones Saghil/Hungary	8.49e-13*	4.199e-12*	1.923e-08*	0.008345*	9.951e-10*

**Table 2: Comparative analysis of the quality of DNA extracted from petrous bones and teeth, of archaeological and/or forensic origin**

\* p-value inferior to 0.05