A comparison of ancient DNA yields across ossicles and the petrous bone reveals the best preservation in the stapes and incus

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Abstract

The petrous bone is considered the most efficient source of endogenous DNA across skeletal tissues in ancient DNA (aDNA) research as well as in forensic work. Recently, ancient DNA in auditory ossicle bones was shown to be comparably well-preserved as in the petrous, although no attempt was made to distinguish among the three ossicle bones. In this study, we compare aDNA profiles across matched ossicle- and petrous-derived sequencing libraries prepared from 29 human skeletons from Neolithic Anatolia and Medieval Iberia. We find that the stapes and incus provide higher human endogenous aDNA than the petrous bone, with >2× higher median rates of endogenous aDNA recovery, while the malleus performs similarly to the petrous. Human aDNA fragments retrieved from the stapes were 8% longer than those from the petrous, whereas postmortem damage, clonality and contamination rates were comparable among the studied bone types. These observations are corroborated by data from non-matched ossicle or petrous libraries from 81 individuals from the same contexts, with the highest endogenous aDNA content observed in the stapes. Despite being the smallest bone in the human skeleton, the stapes, along with the incus, may be among the most optimal aDNA sources yet identified.

Introduction

The efficient retrieval of postmortem or ancient DNA (aDNA) from archaeological and historical human skeletal remains is a major limiting factor in paleogenomics and forensic genetics. Over the last decade, the petrous bone has been recognised as the most effective source in both archaeological and forensic settings (Gamba et al., 2014; Gaudio et al., 2019; Pinhasi et al., 2015, 2019). Endogenous human DNA preservation in the petrous bone (and more specifically, the osseous labyrinth, or the otic capsule, which includes the cochlea) tends to be better than in other skeletal elements, including long bones (femur, tibia etc.), phalanges, teeth, calcaneus, and the talus (Haarkötter et al., 2023; Hofreiter et al., 2021; Parker et al., 2020; Zupanič Pajnič et al., 2025). This property is attributed to limited bone

remodelling that occurs during lifetime and a high concentration of osteocytes in the petrous (Ibrahim et al., 2022; Pinhasi et al., 2015).

Before the discovery of the petrous bone as an effective human aDNA source, Bell and colleagues had suggested that ear ossicles, which contain a high proportion of mineralised osteocytes, could be an optimal target for DNA and protein retrieval from postmortem material (Bell et al., 2008). More recently, Sirak *et. al*. (2020) compared ancient DNA yields in ossicles and petrous bone from 10 human skeletons by shotgun sequencing. These authors found: a) similar endogenous DNA content in ossicles as in the petrous bone (with non-significantly higher levels in ossicles), b) slightly lower DNA postmortem damage (in the form of cytosine deamination) in ossicles, c) three times higher human contamination in ossicles (even though all the libraries studied had <5% contamination levels), and d) similar levels of library complexity.

Sirak and colleagues thus concluded that ossicle bones could be a useful source of aDNA, especially when the petrous bone is not accessible and/or when its destructive sampling is undesirable (Sirak et al., 2020), as its morphology can be used for studying genetic relationships (Ponce de León et al., 2018). Several paleogenomic and forensic genetic studies have also included ossicles as a DNA source, although with limited sample sizes and/or without comparison with the petrous bone (Prendergast et al., 2019; Schwark et al., 2015). Meanwhile, no study, to the best of our knowledge, attempted to differentiate between the three different auditory ossicle elements: the stapes (stirrup), malleus (hammer), and incus (anvil). These three bones might be expected to differ in DNA preservation levels given the differences in their developmental profiles, bone and osteocyte densities (Ivanovic et al., 2024; Marotti et al., 1998; Richard et al., 2017).

Results

We compared aDNA sequencing libraries obtained from 29 archaeological skeletons with both petrous and ossicle tissue, including 9 stapes, 9 malleus, 8 incus, and 4 unidentified ossicle fragments (**Methods**). The skeletons included adults and subadults, and derive from

four archaeological excavation sites from across ancient Central Anatolia and Upper Mesopotamia, ranging from c.11,000 BP to 7,000 BP, and covering mainly temperate steppe-like environments, and from two sites in Iberia dated to the Medieval period (8th to 13th century CE) (**Supplemental Table S1**). Ossicles were collected from temporal bones that had not been washed (a widespread procedure applied in skeletal anthropology laboratories to clean excavated bones with water, which can cause the loss of ossicles [For representative ossicle bones, see **Figure 1**]).

We removed soil deposits within ear canals, which occasionally revealed ossicle bones (see **Discussion** and **Methods** about retrieval methods and frequencies). When multiple ossicle elements were present, we randomly chose one ossicle bone, except for one individual (Ind078) who was sampled for both malleus and incus (**Table 1, Supplemental Table S1**, **Methods**). We additionally expanded our dataset with 81 unmatched ossicle- or petrous-derived libraries from the same site (Site 7), including 5 stapes, 4 malleus, 2 incus and 1 unidentified ossicle fragment without matching petrous libraries, and 69 petrous-derived libraries without matching ossicle libraries **(Table 1, Supplemental Table S1)**. We further used 5 petrous-matched tooth libraries from Neolithic to Early Modern contexts in Anatolia for microbial content analyses (**Supplemental Table S1**, **Methods**).

The ossicles were dissolved directly in the lysis buffer, while the petrous bone samples were pulverised by drilling, and DNA was extracted using the Dabney protocol (2013) **(Methods)**. We constructed double-stranded Illumina libraries using the Dabney extraction (Dabney et al., 2013) and Meyer-Kircher library preparation protocols (Meyer & Kircher, 2010) for the 122 libraries from Anatolia and single-stranded libraries using the Santa Cruz method (Kapp et al., 2021) for the 26 libraries from Iberia. We shotgun sequenced each library once between approximately 0.8 - 80 million reads (median ~11.5 million). To standardize comparisons, libraries exceeding 1 million total reads were downsampled to a 1-million-read threshold **(Methods; Supplemental Table 1)**. We mapped the data to the human reference genome (GRCh37 and compared various characteristics of the ossicle- and petrous bone-based libraries **(Methods)**: endogenous human DNA proportion, human fragment length,

postmortem damage (PMD), clonality, and contamination. We also compared the microbial content in terms of possible sources, i.e. human, oral microbiome, and environmental microbes, to characterize the microbial profile of each tissue type, which we also compared with 5 teeth libraries (**Methods**).

Endogenous human DNA proportion, the most direct indicator of ancient human DNA preservation, was calculated as the fraction of reads mapping to the human genome (without removing duplicates). Across the combined set of 30 ossicle-based versus petrous-based libraries, there was a visible and significant increase in endogenous human DNA in the former [Wilcoxon signed-rank test (WSR) test $P<0.001$)]. This effect was mainly observed for the stapes and incus **(Figure 2A)**. In 9 petrous-stapes comparisons we found a median gain of ~110% in the stapes (WSR test $P=0.027$). In 7 out of 9 comparisons the stapes had higher endogenous DNA; only in a single individual did we observe a visibly lower yield in the stapes than in the petrous, and in one case the petrous and stapes had equally high (~70%) endogenous DNA. In the petrous-incus comparisons (n=8), we observed an increase in human endogenous DNA values in all cases, with a median increase of ~380% (WSR test $P=0.008$). Among the malleus-petrous comparisons, 7 in 9 cases revealed slightly higher endogenous human DNA than the petrous, although there was no significant effect (median increase ~32%; WSR test $P=0.20$) (**Figure 2A**).

We further compared DNA content across all 140 ossicle and petrous-derived libraries in our expanded dataset (14 stapes, 13 malleus, 10 incus, 5 unidentified ossicle, and 98 petrous, belonging to either the same or to different individuals). In these unmatched comparisons, we found significantly better human DNA preservation in the stapes than in all other studied tissues [Mann-Whitney *U* (MWU) test with Benjamini-Hochberg adjustment for multiple testing, $P_{adj}<0.001$]. The incus had the second best preservation in these unmatched comparisons, with significantly higher values than in the malleus and petrous (MWU test $P_{adj}<0.05$) (**Figure 3A**). To control for variability among archaeological sites in this expanded dataset, we restricted the analysis to libraries derived from subadult skeletons interred in the Neolithic/Chalcolithic levels of Site 7 (11 stapes, 8 malleus, 4 incus, 1 unidentified ossicle,

and 80 petrous libraries). We again found superior performance of the stapes compared to both the petrous and malleus (MWU test P_{adj}<0.05; **Figure 3D**). We then asked whether the observed differences could reflect a pattern where the stapes is retrieved only in highly preserved temporal bones but is lost in low-preserved ones, creating an artificial signal of higher endogenous DNA in the stapes in unmatched comparisons. To test this, we limited comparisons to libraries with human proportion values ≥25% in both the petrous (n=28) and the stapes (n=10). We again observed higher endogenous DNA proportions in the stapes compared to petrous libraries (MWU test P_{adj}<0.001; **Figure 3D**). The same comparisons involving the incus vs. petrous bones were not statistically significant, likely due to the limited number of incus specimens (n=4 and 3, respectively).

Human DNA fragment length is another indicator of DNA preservation. Fragment lengths in the 9 stapes-derived libraries were, on average, significantly longer (mean +5.1 bp) than matched petrous-derived ones (WSR test P=0.01; **Figure 2B**). There was no significant difference between the 8 matched incus- and petrous-derived libraries (WSR test P>0.05), while fragment lengths in 9 malleus-derived libraries were on average slightly shorter than (mean -4.3 bp) than in matched petrous-derived ones (WSR test P=0.01; **Figure 2B**). Across the set of all 140 ossicle and petrous libraries (matched and unmatched), we found trends towards longer fragments (mean +5.6 bp) in the stapes and towards shorter fragments in the malleus and incus (means -3.3 bp and -5.4 bp, respectively) compared to the petrous, but none were significant (**Figure 3B**, MWU test P_{adj}>0.05). A similar pattern was observed when the analysis was restricted to Site 7 individuals across varying human proportion thresholds, with no significant differences between ossicle and petrous libraries (**Figure 3E**, MWU test P_{adj}>0.05).

We next studied PMD, estimated as cytosine deamination-caused C-to-T mismatches on fragment ends. We found similar PMD levels between ossicles and matched petrous libraries for each ossicle type, and also no significant difference in comparisons involving all

140 libraries or when restricting the analysis to Site 7 individuals at varying human proportion thresholds (WSR test P>0.05; **Figure 2C, Figure 3C-F**).

Sirak and colleagues had reported slightly but non-significantly higher clonality (inverse of library complexity) and also significantly higher contamination levels in their ossicle libraries compared to their petrous libraries (Sirak et al., 2020). We measured clonality as the proportion of duplicate reads within each discrete library. Clonalities of ossicle-derived libraries were similar to those of matched petrous ones, with no significant difference (WSR test P>0.05; **Figure 2D**). We further studied human contamination estimates using the *contamMix* method (Fu et al., 2013), limiting the analysis to 18 matched pairs with at least 1× mitochondrial DNA coverage (**Methods**). We found no significant difference in human contamination levels between ossicle- and petrous-based libraries (WSR test P>0.05; **Figure 2E**).

Finally, we analysed the microbial content of the studied petrous and ossicle samples, as well as 5 tooth cementum libraries and 3 matched petrous libraries included for this comparison (**Supplemental Table S2**). We assigned reads to microbial taxa using KrakenUniq (Breitwieser et al., 2018) and further divided these into oral and non-oral categories using several databases (**Methods**). A small fraction of reads were assigned to oral microbes in teeth libraries (median 0.22%) and practically none in petrous and ossicle libraries (median 0-0%; **Supplemental Figure 1A**). Teeth also had the highest proportions of reads assigned to non-oral microbes (median 2.3%; **Supplemental Figure 1B**), which include environmental taxa (e.g., soil and aquatic microorganisms associated with postmortem decomposition and taphonomic processes) as well as potential laboratory contaminants. Petrous libraries ranked second (median 0.32%), while ossicle libraries again contained practically no reads assigned to non-oral microbes (~0.01%; **Supplemental Figure 1B)**). These rates were significantly lower for the stapes and incus compared to either the teeth or the petrous (MWU test P_{adj}<0.05).

Discussion

Sirak *et al.* had previously suggested that ossicles could be used as an alternative to the petrous bone as an efficient ancient DNA source (Sirak et al., 2020). Our results now indicate the stapes, and likely the incus, as significantly better sources of human aDNA than the petrous and the malleus. This quality is reflected not only in higher endogenous DNA proportion (for the stapes and incus) but also in the longer DNA fragments (for the stapes). This would potentially elevate the stapes to the top of the list of human skeletal elements for ancient and forensic DNA retrieval.

Our conclusions are subject to a number of limitations. First, we could not conduct within-individual comparisons across the three ossicle types, as nearly all our petrous-matched samples consisted of one randomly sampled ossicle bone per individual. Meanwhile, both the overall endogenous DNA content (**Figure 3A, Figure 3D**), as well as the improvement of the endogenous DNA content relative to the petrous (**Figure 2A**), indirectly support higher performance of the stapes and the incus over the malleus. In the single case where we could study both the malleus and incus from the same individual (Ind078), the incus had >2× higher endogenous content (33% vs. 73%, respectively), in line with the indirect comparisons.

The availability of the ossicles from skeletal collections and preservation biases poses another question. In our preliminary records, the relative frequency of ossicle detection varies significantly among sample sets. Across 566 Anatolian skeletons studied (by D.D.K.) spanning the last 10,000 years, we recorded at least one ossicle (without distinguishing between types) in 52 instances (9%). Notably, the majority of these skeletons were washed to various degrees, and the lowest frequency was recorded in the Neolithic period (c.9000-6000 BCE) Anatolian sample (4 in 147). In the Medieval Iberian sample, which was also heterogeneous with respect to cleaning state and included 70 petrous bones (studied by G.O.G.), we identified at least one ossicle 48 times (69%); the retrieval frequency again seemed to be inversely related to the petrous bone being washed. These figures are compatible with reported ossicle retrieval frequencies in the literature, ranging from 4% to 53% in diverse collections (Bruintjes et al., 1997; Qvist, 2000; Sakalinskas & Jankauskas,

1991). This variation in ossicle retrieval is partly attributable to care in excavation, storage, and processing (Qvist, 2000), including whether washing is applied or not. Taphonomic processes likely also play a role, reflected in the high (>50%) ossicle retrieval frequencies reported for Medieval Iberia and Denmark [this study and Qvist (2000), respectively]. In addition, in four publications reporting ossicle retrieval, the stapes was found at lower frequencies (30-50%) relative to the incus and malleus (Bruintjes et al., 1997; Lisoněk et al., 1986; Qvist, 2000; Sakalinskas & Jankauskas, 1991). Qvist (2000) suggested the difference could represent the erosion of the stapes during lifetime. Such age-related fragility could also explain why most of the stapes samples in our study derived from Anatolian Site 7, which was dominated by newborns (**Supplemental Table S1**). It is further possible that ossicles, and especially the stapes, are often found when the overall skeletal preservation is good. An observation that supports a preservation bias is that in our sample from Site 7, non-matched petrous libraries (n=69) have much lower endogenous DNA proportions than the matched petrous libraries where ossicles were identified (n=11): median 7% vs. 54%. We remind, however, that even among highly preserved samples, the stapes had better DNA yields than the petrous. Future work systematically documenting the frequency of ossicle retrieval in diverse settings and among carefully handled crania, including the frequency where they might be found in both left and right temporal bones, could help further assess the potential of the use of ossicles as an aDNA source.

Another potential limitation of our study involves the efficiency of petrous bone sampling. We extracted DNA from the petrous bone by drilling into the otic capsule and mainly targeting the cochlea, but we frequently also drilled the vestibule and semi-circular canals in order to collect additional power. This may have introduced variability in aDNA retrieval, as the cochlea is considered the most dense element (Pinhasi et al., 2015) and the heat caused by drilling (even though generally performed at low speeds) could degrade biomolecules. The protocol Sirak *et al*. (2020) adopted in their petrous versus ossicle comparison was more targeted, where the authors isolated the cochlea using a sandblaster and powderized the bone with a freezer mill (Pinhasi et al., 2019). This latter approach is possibly more efficient

246 in retrieving aDNA from the petrous bone and might partly explain why we observe improved
247 rates with ossicles over the petrous, while Sirak and colleagues found comparable rates.
248 However, this likely does not explain all our findings because we also observe differences
249 among the ossicle types. For instance, we find that the endogenous DNA proportions in the
250 malleus are comparable to those in the petrous, similar to what Sirak *et al*. (2020) report for
251 ossicles in general, and they are lower than those of the stapes and incus. Further, human
252 aDNA fragments in the stapes tend to be longer than in other tissues. Considering that our
253 ossicle processing approach, dissolving in an incubation buffer, is the same as that of Sirak
254 and colleagues, the higher performance of the stapes relative to the petrous bone and the
255 malleus is unlikely to be fully explained by petrous drilling.
256 Finally, we did not include teeth in our paired comparisons of endogenous proportion.
257 Previous reports have found the tooth cementum to perform either similar to the petrous
258 bone (Hansen et al., 2017) or slightly worse (Parker et al., 2020) in terms of human DNA
259 preservation. We can thus infer that the stapes and incus would have higher endogenous
260 DNA yields than the cementum. Meanwhile, teeth samples have the advantage of carrying
261 information about commensal and pathogenic microbes of diverse origin compared to the
262 petrous bone (Sikora et al., 2025). Our analyses suggest that the ossicles may carry even
263 lower amounts of microbial DNA than the petrous and would not be a useful resource for
264 metagenomic analyses.
265 The ossicle bones are known to undergo only limited remodelling after 1-2 years of age
266 (Marotti et al., 1998; Rolvien et al., 2018), a pattern also observed in the petrous bone
267 (Frisch et al., 1998; Sølvsten Sørensen et al., 1992). Sirak and colleagues (2020) argued
268 that this limited bone remodelling could explain comparable DNA preservation levels in
269 ossicle and petrous samples. In the absence of remodelling, mineralised, apoptotic-like
270 osteocytes with condensed chromatin structures are reported to accumulate in ossicles (Bell
271 et al., 2008; Rolvien et al., 2018), which could be the source of preserved aDNA. Our
272 microbial analyses also imply that the ossicles may be generally more resistant to microbial
273 colonisation than the petrous and tooth. The reason for the relatively good DNA preservation

in the stapes (and possibly the incus) over the malleus is yet unclear. Several studies have reported differences in histology and development among ossicles, including the later ossification of the stapes compared to the malleus and incus (Richard et al., 2017), high bone density in the stapes footplate (Ivanovic et al., 2024), a higher percentage of dead osteocytes in the stapes compared to the other ossicles through lifetime (Marotti et al., 1998), and increasing fragility with age (Qvist, 2000). Such differences could possibly contribute to differential survival of bone structure and of DNA among the ossicles.

Our work indicates that the stapes, with its small size (c. 3-6 mm) and fragile appearance, is an exceptionally preserved bone element. Despite its relatively limited survivorship, whenever retrieved, it can be a superior source of human ancient DNA over the petrous as well as other human bone elements. The incus may also be a comparably good source, at least with respect to endogenous DNA proportions. Relative to petrous sampling, the ossicles have the additional advantage of being amenable to collection for aDNA analysis without destruction to the temporal bone. Meanwhile, the ossicles also have a unique role in bioarchaeology because they can be used to study maternal diet during pregnancy through stable isotopes (because of their low postnatal remodelling). However, the stapes has been noted as being too small for sufficient collagen extraction for dietary analysis (Leskovar et al., 2019). Sampling the stapes for aDNA analysis therefore appears a reasonable choice, notwithstanding the two constraints mentioned earlier: its relatively bad preservation (compared to the petrous bone) and its lack of metagenomic information about commensal or pathogenic microbes (compared to teeth) (Margaryan et al., 2018; Sikora et al., 2025). Beyond these limitations, the stapes could become a main target tissue for archaeogenomic studies and also in forensic analyses (Zupanič Pajnič et al., 2025).

We thus echo the call by Sirak and colleagues to the archaeology and bioarchaeology communities to treat skull fragments carefully to avoid the loss of ossicles during excavation and to refrain from washing temporal bones before sampling ossicles. For ossicle retrieval protocol from archaeological skeletons, see **Methods**.

Methods

All experiments were conducted in dedicated ancient DNA laboratories at Middle East Technical University and Hacettepe University in Ankara, Türkiye, and the Center for Paleogenetics of Stockholm University, Sweden. Anatolian skeletal material was processed in Ankara and Iberian material in Stockholm. Experiments were performed by five researchers (listed in **Supplemental Table S1**).

Three features of the experimental design limit possible confounding between biological and technical artefacts: 1) our main observations were based on matched comparisons between ossicle- vs. petrous-derived libraries, 2) in matched comparisons, we used the same set of reagents for the petrous and the ossicle libraries, 3) in unmatched comparisons, the bulk of the ossicle and petrous libraries derived from a single archaeological context (104 in 140, Anatolian Site 7) and were produced by a single researcher with the same reagents (produced within 08/2021-07/2022), allowing us to perform unmatched comparisons using only this data subset.

Several precautionary measures were taken to minimise the risk of contamination during the experiments. All equipment, utensils and laboratory surfaces were decontaminated with DNA AWAY (Thermo Fisher) or a bleach solution before each use and in-between different samples. All solutions not sensitive to UV light were exposed to UV-irradiation for 30 minutes in a UV crosslinker. Negative controls were included at every step necessary to monitor potential contamination.

Sample preparation

Ossicle sampling. Ossicles were collected from temporal bones where the middle ear had not been fully cleaned by washing. The procedure requires careful handling as outlined in a video protocol (dx.doi.org/10.17504/protocols.io.kxygx42zol8j/v1). Briefly, soil deposits in the ear canals were removed with cotton swabs and/or picks, using distilled water depending on the soil consistency, which occasionally revealed ossicles dislodged in the process. Sometimes, we also found the stapes fallen into the window opening leading to the

vestibule. From ossicles collected in this manner from each temporal bone, we randomly selected one element (except for Ind078 sampled for both the malleus and incus). We analysed only one temporal bone per individual, such that the petrous and the ossicle samples derived from the same side, right or left. A small number of samples collected in the early stages of the experimental work when we had not started recording the ossicle type and a few cases where the ossicles were too fragmented to preclude identification were collectively labeled as "unidentified ossicle". In Ankara, the ossicle bones retrieved were used directly in the following steps, while in Stockholm, they were decontaminated when soil deposits were present on the bone surface by wiping with cotton swabs soaked in 1% bleach solution, followed by rinsing with distilled water.

Petrous sampling. Ear canals within the petrous bones were cleaned both mechanically and chemically when extensive soil deposits were present. Mechanical cleaning involved removing the outer layer of the ear canal using Dremel tools with a sterile tip, whereas for chemical cleaning we used 1% bleach solution followed by rinsing with sterile water.

From the petrous bone (otic capsule) material, we obtained approximately 20-80 mg of bone powder by drilling into surface-cleaned bones with a Dremel 3000 or Dremel Fortiflex 9100-21. The drilling targeted the otic capsule, which primarily includes the majority of the vestibule; however, the semi-circular canals and cochlea were also occasionally included in order to collect sufficient powder from the petrous bone. In experiments performed in Ankara, the drilling was performed at the lowest speed (approximately 5,000 RPM for the Dremel 3000 and near 0 for the Dremel Fortiflex 9100-21) with minimal contact time to reduce heat. In experiments performed in Stockholm, drilling was performed at medium speed.

Tooth sampling. The tooth samples used in this study date to Neolithic, Medieval and Early Modern contexts from Anatolia **(Supplemental Table S1)**. Molar teeth that were intact within the mandible or maxilla were used in this study. In the first step, teeth were removed from the alveolar bone and cleaned with distilled water using cotton swabs or towels if needed.

Subsequently, the pulp cavity was drilled at the level of the cementoenamel junction using a Dremel tool as described for petrous bone sampling.

Experimental procedures

DNA extraction. We used a modified protocol based on Dabney et. al. (2013) for ancient DNA extraction. The auditory ossicle bones were directly added to the lysis buffer, without pulverising or additional cleaning. Ossicles or petrous bone powdered samples were incubated in 1 ml of lysis buffer (0.45M EDTA and 0.25 mg/ml Proteinase K, pH:8) in screw tubes at 37°C in a rotor incubator. Tooth samples were directly immersed in a lysis buffer (approximately 2-3 mL) in 15 mL centrifuge tubes and incubated under the same conditions as the ossicle and petrous bone samples.

After the lysis buffer was saturated (approximately 18-48 hours), we centrifuged the tubes and transferred the supernatants into new clean tubes without disturbing the pellet. An additional 1 ml of lysis buffer (2-3 mL of lysis buffer used for tooth samples) was added onto the tubes and incubated again at 37°C till the buffer saturated. After the second incubation, the tubes were centrifuged at maximum speed and the supernatants from the two digestion steps for each sample were combined. Next, we added 13 ml of freshly prepared binding buffer (pH:5.2, 5M Guanidine Hydrochloride, 40% (vol/vol) Isopropanol, 0.05% Tween-20 and 90 mM Sodium Acetate) onto the supernatants and filtered the mix through MinElute Silica Columns (Qiagen). Silica columns were washed twice with a 750 µl PE buffer (Qiagen). At the final step, 50 µl of EB buffer (Qiagen) was added onto the filters in silica columns for DNA elution and the procedure was repeated twice.

Library preparation and indexing. For libraries prepared in Ankara, we followed the Meyer and Kircher (2010) protocol for preparing Illumina-compatible double-stranded libraries. Blunt-end repair of aDNA fragments was performed with T4 DNA polymerase (Thermo Fisher Scientific), followed by the ligation of adapter oligos to the repaired fragments using T4 DNA Ligase (Thermo Fisher Scientific). Next, the flanking regions of the adapters were filled in using the enzyme Bst Polymerase, Large Fragment (New England Biolabs). We then

purified the libraries using the Qiagen MinElute PCR kit. To check the quality of the double-stranded libraries, we performed qPCR analysis. The qPCR Ct values were used to estimate the number of PCR cycles necessary to amplify each library to the threshold value. Each library was amplified and dual-indexed with Illumina-compatible indexes in three 50 µl PCR reactions (Kircher et al., 2012) using AmpliTaq Gold 360 (Thermo Fisher Scientific). From the three PCR products we collected ~150 µl from each and pooled these together in a single low-binding microcentrifuge tube. To remove primer dimers, non-ligated adapters, and long fragments likely to represent modern contamination, and to achieve homogeneous fragment sizes, we then cleaned the libraries using AMPure XP beads (Beckman Coulter), sequentially at 0.5× and 1.8× ratios. The concentrations of purified libraries were measured with the Qubit dsDNA High Sensitivity Kit. The fragment length and concentration of the libraries were also assessed using an Agilent 2100 Bioanalyzer DNA High Sensitivity Kit.

For libraries prepared in Stockholm, we used the single-stranded Santa Cruz Reaction protocol (Kapp et al., 2021), with modifications from Nguyen et al. (2023). The libraries were dual-indexed as described above. They were then purified and cleaned using Cytiva beads at specific ratios (0.5× and 1.7×). Library quality was assessed on an Agilent TapeStation with D1000 screen tapes.

For sequencing, libraries with unique index pairs were pooled in equimolar concentrations (final concentration of 10 nM total pool) and sequenced 2×150 cycles on Illumina NovaSeq 6000 S1/S4 or NovaSeq X platforms at the National Genomics Infrastructure in Stockholm. Each library was sequenced once.

Data analysis

Data preprocessing and downsampling. Adapter sequences were removed using "*-qualitybase 33 -gzip -trimns*"' parameters using the AdapterRemoval (v2.3.3) (Schubert et al., 2016). Paired-end libraries were merged after removing residual adapter sequences, requiring at least 11 bp overlap between the pairs using additionally "*-collapse* -

412 *minalignmentlength 11*". The quality of the sequences were studied using the FASTQC
413 software (*https://www.bioinformatics.babraham.ac.uk/projects/fastqc/*).
414 We performed the following downsampling scheme to obtain FASTQ files for each library
415 with ~1 million raw reads (including human and non-human reads) for comparable estimates
416 of endogenous proportion and clonality, while keeping human mitochondrial DNA reads up
417 to 3× mtDNA coverage in order to obtain reliable contamination estimates using the
418 contamMix software (Fu et al., 2013). a) We mapped reads in the full libraries to the human
419 reference genome (GRCh37) using the "*aln/samse*" algorithm of the Burrows-Wheeler
420 Alignment (BWA) software (v 0.7.15) (Li & Durbin, 2009) with parameters "*-n 0.01, -o 2*" and
421 by disabling the seed with "*-l 16500*". We used GRCh37 out of convention, as this genome
422 version has been used as the main human reference in ancient genome studies to date.
423 Although using GRCh38 or T2T would increase proportion of reads mapped, this is not
424 expected to alter the average differences between petrous and ossicle libraries in terms of
425 endogeneous proportion or length statistics (Sirak et. al., 2020; Mallick et. al., 2024). b) We
426 subset the BAM files with reads mapping to the mtDNA using SAMtools software (v 1.18) (Li
427 et al., 2009) with "*view -b MT*" parameters, then downsampled these up to 3× mitochondrial
428 coverage using SAMtools software (v 1.18) (Li et al., 2009) with "*view -b -s <fraction_value>*"
429 parameters. Utilizing the read IDs from the resulting files, mitochondrial reads were extracted
430 from the pre-processed FASTQ files using SeqKit software (v2.11.0) (Shen et al., 2024) with
431 "*grep -f <read_ID_list>*" parameters. This procedure yielded FASTQ files containing
432 exclusively mitochondrial reads, limited to a maximum mitochondrial genome coverage of
433 3×. c) We downsampled the preprocessed FASTQ files to 1 million reads using the seqtk
434 software (v1.3-r106) (*https://github.com/lh3/seqtk/tree/v1.3*) with "*sample -s150 1000000*"
435 parameters. d) To exclude mitochondrial sequences from the downsampled FASTQ files, the
436 data were re-aligned to the human reference following the same parameters in step (a).
437 Mitochondrial read IDs were then identified using SAMtools (v 1.18) (Li et al., 2009) with the
438 "*view MT*" parameters. Finally, these mitochondrial reads were removed from the
439 downsampled FASTQ files using SeqKit (v2.11.0) (Shen et al., 2024) with "*grep -v -f*

<*read_ID_list*>" parameters. This procedure resulted in FASTQ files subsampled to 1 million reads, entirely devoid of mitochondrial sequences. e) We merged FASTQ files resulting in the steps (b) and step (d). We obtained the total number of sequenced reads from these merged FASTQ files. f) Finally, we re-aligned the merged FASTQ files to the human reference using the same parameters as in (a).

Calculating mapping statistics. We calculated the human proportion using the BAM files obtained as a result of this alignment process. PCR duplicates with identical start and end coordinates were removed using the script "FilterUniqueSAMCons.py" (Kircher, 2012). To minimise spurious alignment, reads with alignment quality of less than 30, shorter than 35 base pairs, and containing more than 10% mismatch were discarded. After alignment, we calculated the average genome coverage using the genomeCoverageBed algorithm implemented in the bedtools2 software (v2.31.0) (Quinlan & Hall, 2010) with reads with a mapping quality >30. The aligned libraries were examined using the PMDtools algorithm with the "*-deamination*" parameter to calculate postmortem damage in DNA (v0.60) (Skoglund et al., 2014). The base position was determined where the average postmortem damage (cytosine to thymine deamination) at the 5' ends of reads in each library falls below 1%. The distribution of read lengths and contamination probabilities based on mitochondrial DNA diversity were calculated with the contamMix algorithm using default parameters (Fu et al., 2013). To ensure the reliability of contamination estimates, the latter analysis was restricted to petrous and ossicle-matched libraries both having at least 1× mitochondrial DNA coverage (contamMix estimates become unreliable below this range).

To determine the human endogenous DNA content of each obtained library, we used the ratio of the sequences (i.e. merged reads) aligned to the human reference genome to all sequences in that library. We used the filtered sequences (as described above) but without removing duplicates (because technical factors such as higher PCR cycles or sequencing depth will increase duplication levels).

To calculate mean fragment lengths for raw reads from FASTQ files, we used SeqKit (v2.11.0) (Shen et al., 2024) software with the "*stats -T*" parameters. For mapped reads, we used samtools with a "*-q 30*" filter to obtain the distribution of fragment lengths from BAM files. We then calculated the mean fragment length for each BAM file using an in-house bash script. Mean fragment length distributions of raw (FASTQ) and mapped (BAM) reads for libraries from each tissue type are summarized in **Supplemental Figure 2.**

To estimate the percentage of clonality, which we define as the proportion of duplicate reads to total reads, was calculated for each BAM file using the same procedure described above.

Metagenomic analysis. For metagenomic screening, we followed the aMeta pipeline (Pochon et. al, 2023) up to the KrakenUniq step. FASTQ files for each library were screened for microbial reads using the KrakenUniq (v1.0.4) (Breitwieser et al., 2018) database provided by aMeta, which includes NCBI non-redundant NT/NR records (June 2020) comprising archaea, bacteria, viruses, fungi, protozoa, parasitic worms, humans, and several complete eukaryotic genomes (Pochon et al., 2023). KrakenUniq results were filtered by retaining taxa with at least 100 unique *k*-mers and 10 unique reads. Microbial source data were obtained from three databases: Expanded Human Oral Microbiome Database (eHOMD v4.1) (Escapa et al., 2018), BacDive Database (Schober et al., 2025) and MicrobeAtlas Database (Rodrigues et al., 2025). Using a custom R script, microbial species were classified as originating from human, oral, or non-oral sources. Relative abundances were calculated by dividing the number of reads assigned to each microbial species/source by the total number of microbial species/source reads assigned to any species in the individual library by KrakenUniq.

Statistical analyses. All statistical analyses were conducted in R (R Core Team, 2025). We used the packages readxl (Wickham & Bryan, 2023), tidyverse (Wickham et al., 2019), and reshape2 (Wickham, 2007) for data processing. Wilcoxon signed-rank tests were performed using the wilcox.test function from the R *stats* package (R Core Team, 2025). Pairwise comparisons were performed using Mann-Whitney *U* tests via the pairwise.wilcox.test function in the R *stats* package (R Core Team, 2025). To control the false discovery rate, p-

values were adjusted using the Benjamini-Hochberg method (p.adj = "BH"). Visualizations were created using ggplot2 (Wickham, 2016), ggtern (Hamilton & Ferry, 2018), superb (Cousineau et al., 2021), rnaturalearth (Massicotte & South, 2023), raster (Hijmans, 2023), ggrepel (Slowikowski, 2024), ggpubr (Kassambara, 2023), ggforce (Pedersen, 2024), ggbeeswarm (Clarke et al., 2025), png (Urbanek, 2022), grid (R Core Team, 2025), ggh4x (Brand, 2024), *DescTools* (Signorell, 2024) R packages.

Ethical considerations

All samples used in this study are archaeological/historical specimens older than 200 years and therefore are not considered human-subject research under current ethical and legal frameworks. As such, institutional ethical approval was not required. The specimens were obtained from authorized collections through the relevant museum/curatorial authorities, and all permissions for sampling, destructive analysis, and publication were granted prior to the study. No modern individuals or identifiable personal data were involved. All work was conducted in accordance with the ethical guidelines for the study of historical human remains of the country of origin.

Data Access

The DNA sequences reported in this paper have been submitted to The European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena/) under the accession number PRJEB108518.

Code Availability

All R codes related to the statistical analyses of this project are available in the following GitHub repository: https://github.com/ardasevkar/Auditory-Ossicles. Additionally, the complete code is provided as a Supplemental Code file.

Competing Interest Statement

The authors declare no competing interests.

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TABLES

Experimental Design	Tissue Combination	Number of Libraries	Number of Individuals
Matched petrous-ossicle samples	P, S	18	9
	P, M	16	8
	P, I	14	7
	P, M, I	3	1
	P, UIO	8	4
	Total	**59**	**29**
Unmatched samples	P	69	69
	S	5	5
	M	4	4
	I	2	2
	UIO	1	1
	Total	**81**	**81**

Table 1: Number of libraries and individuals used. “Matched petrous-ossicle samples” refers to cases where we collected one petrous and one ossicle type per individual (except for a single case where we generated libraries for both malleus and incus from the same skeleton, Ind078). “Unmatched samples” refers to cases where an individual was represented by either a petrous- or ossicle-derived library. P: petrous, S: stapes, M: malleus, I: incus, UIO: unidentified ossicle. The unidentified category includes ossicles where the type had not been recorded or that were fragmented so that morphological identification was not possible. The table does not include five tooth-derived and three matched petrous-derived libraries used in microbial comparisons (two of the tooth-derived libraries have matched petrous and ossicle samples represented in the table). See **Supplemental Table S1** for details of the libraries.

FIGURE LEGENDS

Figure 1: Representative auditory ossicle bones used in this study. Each black and white segment in the scale bar represents 2 millimetres.

Figure 2: Comparison of the mapping statistics in matched shotgun DNA libraries prepared from each ossicle type relative to those prepared from the petrous bone. A) Comparison of human proportions, B) comparison of mean fragment lengths, C) comparison of deamination values (postmortem damage), D) comparison of clonality values, E) Contamination was estimated using the contamMix method (Fu et al., 2013), including only matched libraries with >1× mtDNA coverage. Only statistically significant Wilcoxon signed-rank (WSR) test results are shown (*: P_{adj}<0.05 **: P_{adj}<0.01; ***: P_{adj}<0.001). “UI Ossicle”: unidentified ossicle. “PMD”: postmortem damage.

Figure 3: Comparison of mapping statistics in the expanded dataset. Panels A, B, and C represent mapping statistics for all 140 ossicle- and petrous-derived libraries, whereas panels D, E, and F represent mapping statistics for libraries prepared from subadult individuals recovered from the Neolithic/Chalcolithic levels of Anatolian Site 7 (n=92). Mann-Whitney U test results are indicated above the plots. Only statistically significant comparisons are shown (***: P_{adj}<0.001; **: P_{adj}<0.01; *: P_{adj}<0.05). “PMD”: Postmortem damage. “HP”: Endogenous human DNA proportion.