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ABSTRACT

Horses and donkeys have had a far-reaching impact on human history, providing mechanical power for agriculture and transportation. Their F1-hybrids, especially mules, have also been of considerable importance due to their exceptional strength, endurance and resistance. The reconstruction of the respective role that horses, donkeys and mules played in past societies requires prior identification of their osseous elements in archaeological assemblages. This, however, remains difficult on the basis of morphological data alone and in the absence of complete skeletal elements. While DNA sequencing provides almost certain identification success, this approach requires dedicated infrastructure and sufficient ancient DNA (aDNA) preservation. Here, we assessed the performance of a cost-effective alternative approach based on geometric morphometric (GMM) analysis of the bony labyrinth, a structure carried within the petrosal bone. This extremely compact osseous structure provides good aDNA preservation and is frequently found in archaeological assemblages. To assess the GMM performance, we first used High-throughput DNA sequencing to identify 41 horses, 24 donkeys, 36 mules and one hinny from 11 archaeological sites from France and Turkey spanning different time periods. This provided a panel of 102 ancient equine remains for micro-computed tomography (microCT) and GMM assessment of the variation of the bony labyrinth shape, including the cochlea and the semicircular canals. Our new method shows good-to-excellent prediction rates (85.7%–95.2%) for the identification of species and hybrids when considering the cochlea and semicircular canals together. It provides a cheap, non-destructive alternative to aDNA for the taxonomic identification of past equine assemblages.

1. Introduction

The domestication of the horse and the donkey has dramatically impacted the evolutionary trajectory of past human societies (Blench and MacDonald, 2006; Clutton-Brock, 1992; Kelekna, 2009). Horses provided unprecedented and exceptionally-fast transportation, which fueled long-distance migration (Allentoft et al., 2015; Haak et al., 2015) and facilitated both genetic and cultural exchange (Jeong et al., 2020). Horse-drawn chariots, mounted raids and cavalries have also represented crucial developments in the history of warfare (Drews, 2004), which made horses an essential part of military strategies up until the 20th century (Singleton, 1993). In contrast to horses, donkeys are not

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especially fast animals; they, however, show exceptional resistance and carrying capacities, and are particularly adapted to arid and semi-arid deserts (Maloiy et al., 2009; Marshall, 2016). They have therefore been extensively used as pack and draught animals along important historical commercial routes, such as the trans-Sahelian and trans-Saharan roads (Blench, 2004; Blench and MacDonald, 2006), and remain critical for the economies of many developing countries today.

Together with horses and donkeys, their first-generation hybrids, especially mules (the offspring of a horse mare and a donkey jack) have represented an additional important source of mechanical power in ancient societies. Despite being almost always infertile themselves (Benirschke and Ryder, 2010; Steiner and Ryder, 2013), these animals are more sure-footed, resistant and live longer than both their parental species, and can carry heavier loads for longer distances (Baranwal et al., 2012; Lippman and Zamir, 2007; Lynch, 1991). They have been central to Roman military logistics for transporting goods and weapons across an empire that stretched over thousands of kilometers (Armitage and Chapman, 1979; Granado et al., 2020). Multiple lines of evidence have shown that hybrids were in fact already used in earlier time periods. For example, DNA-based analyses have unambiguously identified mules at sites as early as the La Tène Iron Age (Fages et al., 2019). Mules and hinnies were also mentioned on Mesopotamian cuneiform tablets and seals dated to the first half of the second millennium BCE (Before Common Era; Michel, 2002). The earlier textual reference to equine hybrids in Mesopotamian clay tablets dating back to the third millennium BCE is often interpreted as the cross between a donkey (*Equus asinus*) and an hemione (*Equus hemionus*). In short, the practice of interspecific cross-breeding has been practiced for extensive periods of time and this millenary knowledge, still ongoing today, underlines the importance of such hybrids for human societies.

No matter how much horses, donkeys and mules have driven past societies and economies, their identification as part of archaeological animal assemblages remains difficult. Linear measurements of post-cranial elements often result in the assignment of smaller-sized specimens to donkeys on the basis of the limited size found amongst the modern breeds of this species (Arbogast et al., 2002), or mules when combined to horse morphological characteristics (Hanot et al., 2017). While complete skulls generally show better predictive success rates (Azzaroli, 1978; Eisenmann, 1986; Groves and Mazák, 1967; Hanot et al., 2017; Kunst, 2000), they are, however, relatively rare in archaeological assemblages. The reliability of some more frequent post-cranial elements for species identification has been questioned, especially for mules in which up to 91.7% (11/12) mis-classification rates have been recently reported (Granado et al., 2020). Applying GeoMetric Morphometric (GMM) analysis to a large panel of osteological features, Hanot and colleagues have recently achieved improved classification success on modern reference panels (Hanot et al., 2017). Their methodology, however, shows best performance when combining several bone elements together, which precludes application to those extremely fragmentary records, yet, representing the vast majority of archaeological assemblages. Additionally, true prediction rates could not be assessed outside of modern reference panels as the archaeological remains investigated were not subjected to DNA analysis, which represents thus far the only method allowing sex and equine taxonomic classification, including for hybrids (Schubert et al., 2017).

Previous work has shown that no more than 1,000–10,000 ancient DNA sequences spread across the horse genome can ensure 100% assignment taxonomic success of equine remains (Schubert et al., 2017). While generating such amounts of data is within reasonable reach for the vast majority of specimens showing even moderate DNA preservation, it remains, however, infeasible for those specimens showing no to minimal preservation. Additionally, the methodology is destructive and can only be implemented at reasonable costs for those laboratories equipped with ancient DNA facilities and providing technical support staff. In the recent years, the pars petrosa (often referred to as the petrosal bone) has been shown to be associated with ancient DNA preservation rates that are higher than other skeletal parts (Gamba et al., 2014; Pinhasi et al., 2015). This bone has, thus, received massive attention from ancient DNA scholars and has become a significant fraction of the material underlying the sequencing of ancient genomes (see Orlando et al., 2021 for a review). The petrosal bone, however, includes the inner ear, which is formed by the cochlea providing the sensory organ of hearing (Manoussaki et al., 2008), the vestibule and the semicircular canals, both involved in balance (Ekdale, 2016). Those structures have demonstrated taxonomic discriminatory power on modern hominids and ancient hominins, both at the species and subspecies levels (Spoor et al., 2003; Gunz et al., 2012). The 3D shape of the cochlea also shows excellent prediction rates (91–93%) for sex determination in modern humans (Braga et al., 2019). As almost fully formed at birth in mammals (Jeffery and Spoor, 2004; Maier, 2013), the inner ear shows only limited variation during the postnatal life of an individual, providing robust results even when different developmental stages are considered in a single analysis. Finally, as it is part of the particularly compact and resistant petrosal bone, the inner ear can often be retrieved from archaeological faunal assemblages.

The reasons why we decided to focus on petrosal bones are threefold. First, petrosal bones generally show increased molecular preservation relative to other bone remains, which therefore increases the odds of successful sex and taxonomic identification on the basis of DNA analyses (Gamba et al., 2014; Pinhasi et al., 2015). Second, petrosal bones have previously been shown to be particularly informative for sex determination in modern humans (Braga et al., 2019) and species determination among Primates (Spoor et al., 2003; Gunz et al., 2012; Braga et al., 2015; Beaudet et al., 2019). Third, petrosal bones are extremely dense and compact, which makes them relatively frequent parts of archaeological assemblages. This is in contrast to complete skulls, which show significant discriminant features amongst most equine taxa (Azzaroli, 1978; Eisenmann, 1986; Groves and Mazák, 1967; Kunst, 2000) but are found in more limited archaeological contexts. Petrosal bones, thus, show a wider practical application range than other morphological methods currently available that are based on the whole skull and various combinations of cranial and post-cranial elements, including that recently reported and associated with encouraging predictive power (Hanot et al., 2017).

The relative ubiquity, coupled with the established prediction power in primates, make the inner ear a good candidate for developing a new, non-destructive and low-cost alternative to ancient DNA allowing sex and taxonomic identification in horses, donkeys and mules. We thus endeavored to quantify the assignment performance of the bony labyrinth 3D shape using GMM and a panel of 102 archaeological equine specimens for which sex and taxonomy could be determined on the basis of shallow sequencing on HTS technologies (Fig. 1). To the best of our knowledge, this study is the first to apply micro-computed tomography (microCT) to equine inner ears obtained from archaeological assemblages. The approach presented here achieves good-to-excellent taxonomic prediction rates for both horses, donkeys and mules, with morphological taxonomical classification matching DNA determination in 85.7%–95.2% of the cases. In addition to being non-destructive, our method is also faster and far-less expensive than ancient DNA analyses because as many as 70 bone remains can be scanned in a few hours.

1. Material and methods

1.1. Material

1.1.1. Archaeological material

We collected a total of 102 well preserved right petrosal bones dated to Antiquity, Byzantine, Medieval and Modern time periods, that were excavated from 10 archaeological sites in France and one archaeological site in Turkey. Information on the respective archaeological contexts is provided below. The total sample set was subjected to ancient DNA analysis and successful sex and species determination based on the

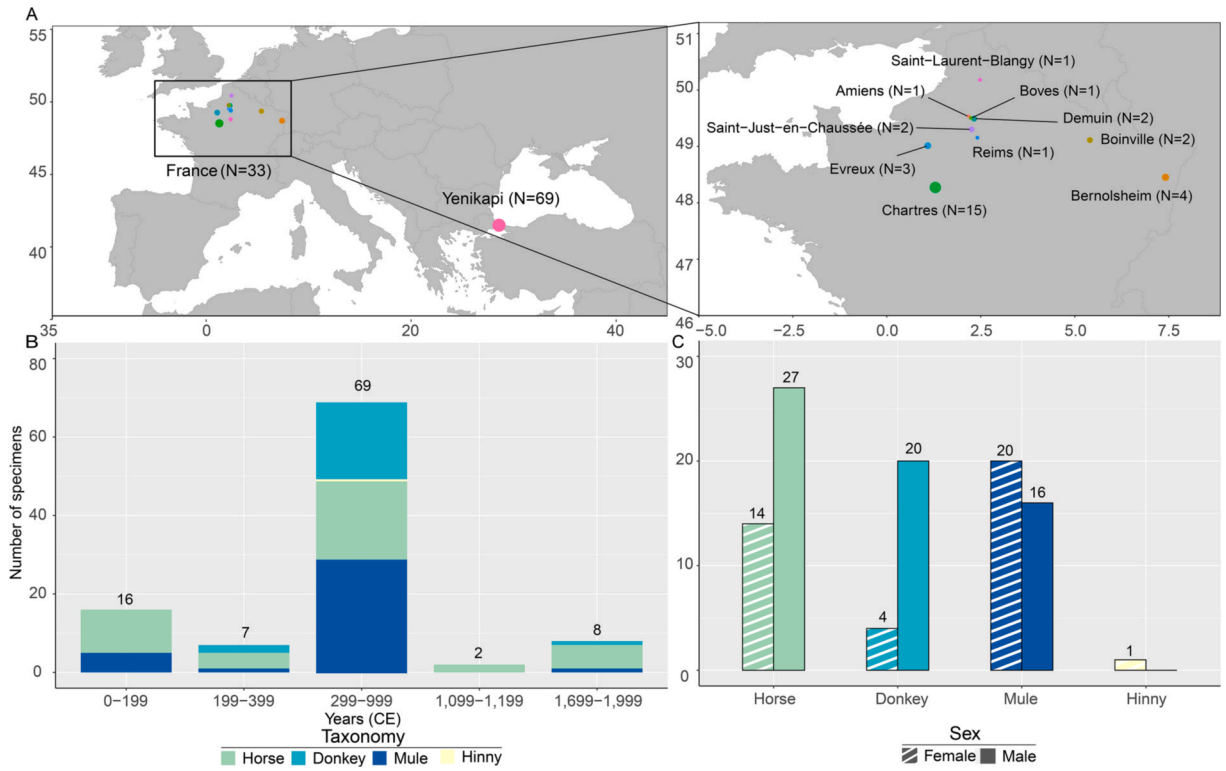

Fig. 1. (**A**) Geographical distribution of the archaeological material collected in Turkey (Yenikapi, N = 69) and France (10 sites, N = 33). (**B**) Temporal distribution of the samples for each taxon (years CE, Common Era) and their genetically-established taxonomic status. (**C**) Sex distribution within each taxon, as determined by ancient DNA.

Zonkey software (Schubert et al., 2017). It included a total of 41 horses (27 males, 14 females), 24 donkeys (20 males, 4 females), 36 mules (16 males, 20 females) and one single female hinny (the offspring of a donkey mare and a horse stallion, i.e. the mule reciprocal cross). Overall, our archaeological material includes 11 archaeological sites, all from France except a single one from Turkey. Their temporal range spans the 1st century CE to the 17th-20th century CE. It is, thus, designed to encompass an entire range of possible individual variation across both parental species and their hybrids.

1.1.2. Archaeological sites

Amiens, Rue Legrand d'Aussy (N = 1, France).

The site was excavated by Josabeth Millereux-Le Béchennec (Service Archéologie Préventive Amiens Métropole, Somme, France) in 2018 and has provided important Roman findings corresponding to the ancient city of Samarobriva (literally, the 'bridge over the Somme' in Gallic). Approximately ~2,000 years ago, this city extended to the present location of the center of Amiens. Following a first phase of indeterminate occupation identified through multiple traces of parceling during the first half of the 1st century CE (Common Era), vast public warehouses organized according to a regular pattern were built around 70 CE but were destroyed during a fire around 120 CE. The site also includes a vast public building built in hemicycle as a theatre. The equine remains analyzed comes from a dump created in this area after the theatre was rebuilt around 160 CE.

Bernolsheim-Momenheim, Plateforme Départementale Activité zone Sud (N = 4, France).

The site of Bernolsheim-Mommenheim (Bas-Rhin, France) was excavated from 2009 to 2014 by staff from the '*Pôle d'Archéologie Interdépartemental Rhénan-Archéologie Alsace*'. The area excavated in 2012 under the direction of Félix Fleischer (including sites 5,645–12,002), yielded large quantities of bones, mainly from a ditch (site 12,002) that was filled by rendering waste dating from the late 17th to the early 20th century CE. The four remains analyzed all came from this ditch amongst several tens of other equids that were excavated.

Boinville-en-Woëvre, Déviation Est d'Etain (N = 2, France).

The site of Boinville-en-Woëvre '*Déviation Est d'Etain*' (Meuse, France) was excavated in 2005 under the direction of Sébastien Viller (*Institut National de Recherche Archéologiques Préventive*, Inrap). It yielded various remains of a Gallo-Roman villa. Approximately ~15 pits containing equine skeletons were discovered within the *pars rustica* area of the villa, which was assigned to agricultural work. These pits could contain multiple animal individuals, either buried simultaneously or separately. The two remains analyzed in this study are part of a larger set of 22 individuals dating to the 2nd-3rd century CE.

Boves « Vallée de Glisy » (N = 2, France).

Excavations at the Boves '*Vallée de Glisy*' (Somme, France) site were carried out in 2009 under the direction of Stéphane Gaudefroy (Inrap). The two samples were excavated within the so-called ST 226 structure, which consists of Roman chalk quarry that was filled during the 3rd century CE and included numerous remains of equids, dogs, sheep, sometimes partly connected.

Chartres, Boulevard de la Courtille (N = 15, France).

Excavations at the '*Boulevard la Courtille*' site in Chartres (Eure-et-Loir, France) were carried out in 2011–2012 by Laurent Coulon (*Service Archéologique de Chartres*). This site corresponds to an ancient residential area, located on the outskirts of the modern city of Chartres. It delivered more than 130 equine skulls arranged between two levels of tracks, both dated to the middle of the 1st century CE. Most of these skulls were remarkably preserved and deposited head-to-tail and oriented perpendicular to the tracks, while the remaining fraction were aligned parallel to the gutter. This has been interpreted as a drainage system (Lepetz et al., 2014).

Démuin, Le château (N = 2, France).

The site of Démuin (Somme, France) was excavated by Richard Jonvel (UnivArcheo) and revealed an occupation extending from the 9th

century CE to the Renaissance. Equine bones represent approximately ~2.8% of over ~5,000 animal remains collected. The two petrosal bones analyzed were discovered isolated within two grain silos that were transformed into dump and dated to the 11th-12th centuries CE (Jonvel, 2014).

Evreux, Parking de l'Hôtel-de-ville (N = 3, France).

The site of Parking de l'Hôtel-de-ville (Evreux, Eure, France) was excavated by Bénédicte Guillot (Inrap) in 2007 and revealed an ancient occupation that lasted until modern times. Starting at the beginning of the 14th century CE, a large traffic area was established on the debris of the earlier buildings. The site showed hardly any occupation during the modern period, which probably explains why local inhabitants used it to bury equine carcasses during the 17th century CE. Age and sex distribution patterns of equine remains indicate the presence of old males. These animals, dead or culled after their use, were recovered by a renderer established at the edge of the city, who took off any useable material (Guillot, 2008).

Saint-Just-en-Chaussée, Secteur 1994–1995 and secteur 1 (N = 2, France).

Excavations at Saint-Just-en-Chaussée (Oise, France) have revealed the presence of both a Celtic sanctuary and a Roman settlement. The so-called 'Secteur 1994/1995' area was excavated during several campaigns by George-Pierre Woimant (Service archéologique départemental de l'Oise) in 1994 and 1995. They revealed several occupations dating from La Tène finale (D1-D2) to the High Empire period. Ditches filled with horse bones, pieces of chariots and harnesses were uncovered. The so-called 'Secteur 1' area has been excavated by François Malrain (Inrap) since 2007 and has yielded numerous Latenian structures including weapons, animal and settlement remains, as well as ritual structures dated back to the Roman period. Among the Roman structures, pit 637 was dated to the 2nd-3rd century CE and yielded large quantities of equine bones, including the two analyzed in this study (Malrain et al., 2017).

Saint-Laurent-Blangy, ZAC Actiparc, Plaine d'Hervin (N = 1, France).

The Saint-Laurent-Blangy site (Pas-de-Calais, France) was excavated in 2002 by Dominique Favier (Inrap), Gilles Prilaux (Inrap) and Alain Jacques (Arras Municipal Archaeological Service). The occupation uncovered lasted from the end of the 2nd century BCE (Before Common Era) to the 4th century CE. The Late Antiquity phase is characterized by the presence of burials and a settlement composed of modest dwellings that have yielded a well from which came the cranial analyzed.

Reims: L'îlot des Capucins-Hincmar-Clovis (N = 1, France).

The Capucin convent in Reims (Marne, France) was excavated by Agnès Balmelle (Afan) and Philippe Rollet (Afan) in 1989. The annex building is located southwest of the cloister and adjoined to the southern dormitory. It included both the kitchens and the laundry. Nine pits were uncovered, all concentrated around the kitchens in an area corresponding to the vegetable garden. Most of these structures were domestic dumps and contained bone food remains. In contrast, kitchen or plate scraps were only residually present in the fill of pit 141, in which the specimen GVA147 analyzed here was excavated. It was dated from the 17th century CE and corresponds to waste resulting from the processing of equine carcasses. The total pit assemblage included only eleven individuals (Minimum Number of Individuals, MNI) and deficiencies in the anatomical distribution profile showed an underrepresentation of lower limbs, most usually the metapodials, indicative that such material was preferentially used by bone workers.

Yenikapi (N = 70, Turkey).

Yenikapi is a site located in present-day Istanbul, Turkey, and was built by Theodosius I during the late 4th century CE. This site was an important harbor for the Byzantine Empire during the Late Antiquity and thus a prominent trade hub in the Mediterranean basin. Excavations revealed an amount of 20,881 bones including those of horses (32.6%), donkeys, mules and hinnies (Onar et al, 2013, 2015bib_Onar_et_al_2015bib_Onar_et_al_2013). A total of 69 specimens were analyzed, including 34 that provided DNA data in previous work (Fages et al., 2019).

1.2. Methods

1.2.1. Ancient DNA analyses

A total of 44 specimens were part of previous ancient DNA work (Fages et al., 2019) and a total of 58 additional ones were analyzed in this study for sex and taxonomic identification. For all specimens newly reported here except Tur 168, Tur177 and Tur179, which were processed at the Centre for GeoGenetics (University of Copenhagen, Denmark) following (Gaunitz et al., 2018), bone pulverization, DNA extractions, library construction and PCR set-up were carried out in the ancient DNA facilities of the CAGT laboratory (CNRS UMR5288), Université Paul Sabatier (Toulouse, France). Sample GVA78 was processed both at the Centre for GeoGenetics and CAGT. Both lab procedures followed conditions strictly limiting DNA contamination, and including Protective Personal Equipment, positive air pressure, bleach and UV decontamination, as well as physical separation of pre- and post-PCR facilities in independent buildings. All laboratory procedures at the Centre for GeoGenetics have been described by Gaunitz et al. (2018). At CAGT, extraction, library construction, amplification and sequencing steps were performed with mock Extraction Blank Controls (EBC) that were subjected to the same treatment as samples in order to detect any contamination. DNA was extracted from 200 to 600 mg bone powder obtained by grinding a portion of the petrosal part of the temporal bone using a ball mill (Retsch MM200). In order to increase the endogenous DNA fraction (Der Sarkissian et al., 2014), the powder was first digested for 1h at 37 °C in 3.85 mL of lysis buffer composed of EDTA 0.5M, N-lauryl Sarcosine (0.5%) and proteinase K 20 mg/mL, following a method developed in previous studies (Gamba et al., 2014; Yang et al., 1998). Following this pre-digestion step and centrifugation at 3,000 rpm (revolutions per minute) for 2 min, the supernatant was discarded, and the recovered pellets underwent a second digestion overnight at 42 °C in an identical volume of fresh lysis buffer. DNA was concentrated and purified from the supernatant fraction of this second digestion by filtration in Amicon Filters (Amicon Ultra 4, 30kD) and successive centrifugations at 3,000 rpm first during 10 min and then 5 min until a final volume of 250 μL was obtained. The supernatant was transferred to a Qiagen Minelute Column with 1.25 mL of PB buffer and centrifugated 1 min at 8,000 rpm. After washing with 600 μL of PE and two centrifugations for 1 min at 11,000 rpm, spin columns were placed in 1.5 mL LoBind tubes (Eppendorf) and DNA was eluted in 60 μL elution buffer (EB-Tween 0.05%, preheated at 37 °C) using centrifugation at 13,000 rpm for 2 min, following 10 min incubation at 37 °C. Volumes of 22.8 μL DNA extracts were incubated with 7 μL USER™ enzyme mix at 37 °C for 3 h following Fages and colleagues (Fages et al., 2019) in order to remove uracil residues and thus reduce the impact of nucleotide mis-incorporations due to post-mortem cytosine deamination, typical of ancient DNA (Rohland et al., 2015).

Except for those four samples processed at the Centre for GeoGenetics and for which single-indexed libraries were constructed, triple-indexed blunt end DNA libraries were constructed following a protocol modified from previous studies (Meyer and Kircher, 2010; Orlando et al., 2013; Seguin-Orlando et al., 2013). We first used the NEBNext End Repair module (New England Biolabs, NEB) with a starting volume of 14.9 μL of USER-treated DNA extract in a total reaction volume of 25 μL and incubation conditions consisting of 20 min at 12 °C followed by 15 min at 37 °C. Then, ligation was carried out using the Quick Ligation module (NEB) in a 25 μL reaction volume at 20 °C for 20 min. Adapters included unique 7 bp-indices that were located at their 3′-end, providing direct library demultiplexing on the basis of the first seven positions within each sequencing read (Rohland et al., 2015). A unique combination was selected to characterize each individual library. Finally, fill-in reactions were performed in 25 μL in presence of the NEB Bst DNA polymerase enzyme and dNTPs (10 mM each), for 20 min at 37 °C and

inactivated for 20 min at 80 °C. Reaction mixes after end-repair and ligation were purified using the MinElute kit (QIAGEN) in 16 μL and 21 μL elution volumes (EB-Tween 0.05% final concentration), respectively. PCR amplification was performed in the following reaction mix using a final volume of 25 μL: 3 μL DNA in solution with EB-Tween, 0.4 μL of AccuPrime DNA polymerase, 2.5 μL of AccuPrime reaction mix (Thermo Fisher Scientific), 1 μL of BSA at 20 mg/mL, 1 μL of 5 μM custom PCR primers one of which containing a unique external 6-bp sequence used as an additional index for sequence demultiplexing (Orlando et al., 2013). PCRs were carried out following 5 min activation at 95 °C, 12 amplification cycles consisting of 15 s of denaturation at 95 °C, 30 s of annealing at 60 °C, 30 s of elongation at 60 °C, and were finished using a terminal elongation of 5 min at 68 °C. Amplified libraries were purified using the Agencourt AMPure XP system (Beckman Coulter) and 35 μL of bead solution. Purified libraries were eluted in 25 μL of EB-Tween (0.05%) and quantified on a 4200 TapeStation Instrument (High Sensitivity D1000 Screen Tape; Agilent) and with a Qubit dsDNA High-Sensivity Assay (Invitrogen). For all samples except GVA78, GVA401, GVA402, Tur169, Tur177 and Tur 170, individual indexed libraries were then pooled together in equimolar proportions and sequenced in 80 bp paired-end mode on the Illumina MiniSeq platform of the CAGT laboratory. Sample GVA78 was sequenced both at the Danish National DNA Sequencing Centre (HiSeq2500 platform, 81-bp single-end mode) and at Genoscope (76-bp paired-end mode). Samples Tur169, Tur177 and Tur179 were sequenced at the Danish National DNA Sequencing Centre (HiSeq2500 platform, 81-bp single-end mode). Finally, samples GVA401 and GVA402 were also sequenced at the Danish National DNA Sequencing Centre (HiSeq 4000 platform, 101-bp paired-end mode and 74-bp paired-end mode, respectively).

Read demultiplexing, adapter removal and quality filtering DNA reads were performed using AdapterRemoval v2.3.0 (Schubert et al., 2016) and mapped against the horse reference nuclear genome (EquCab2 (Wade et al., 2009)) and mitochondrial genome (Genbank accession number NC_001640 (Xu and Arnason, 1994)) using PALEOMIX (Schubert et al., 2014), with default parameters, except that the minimal mapping quality was set to 25 and that the local sensitive mode of Bowtie 2 (Langmead and Salzberg, 2012) was used instead of BWA (Li and Durbin, 2010), following Poullet and Orlando (2020). To establish the authenticity of ancient DNA data recovered, rates of terminal fragmentation patterns and nucleotide misincorporation were evaluated using mapDamage2 (Jónsson et al., 2013). Species and sex determination were performed using the Zonkey package, which is part of PALEOMIX (https://github.com/MikkelSchubert/paleomix) (Schubert et al., 2017).

2.2.2. Acquisition of microCT data and 3D geometric morphometrics

Supplementary video related to this article can be found at https://doi.org/mmcdoino

Right petrosal bones from 102 individuals were scanned at the '*Institut de Mécanique des Fluides de Toulouse*', France (IMFT, N = 100) as well as at the '*Centre Interdisciplinaire de Recherche et d'Ingénierie des Matériaux*' de Toulouse, France (CIRIMAT, N = 2) using X-ray microtomography with a scan resolution ranging between 0.033 and 0.066 mm (EasyTom XL Micro, RX solutions). A total of 94 specimens were scanned following DNA analysis, whereas nine petrosal bones were first processed for microCT and then for DNA. All specimens provided sufficient DNA material for sex and taxonomic identification.

MicroCT scans were first imported into the Avizo v8.1 software package in order to carry out segmentation and 3D reconstruction of the bony labyrinth. The segmentation step consisted in the filling of the canal network that constitutes the inner ear structure. A first observation of orthogonal plans allowed separating the cochlea and semicircular canals in order to conduct downstream analyses in which both structures were considered altogether or separately. To characterize these structures, markers were placed every 10 slices with the surface editor, and filling was carried out using the watershed tool. We next generated the surface with light smoothing and obtained 3D models. Among the 93 samples drilled prior to CT-scanning, 20 bony labyrinths showed damages caused by intrusive sampling, which affected both the common cross and the base of the posterior and anterior semicircular canals. Downstream analyses were thus limited to the preserved structures on these and the remaining 82 samples (N = 102), but were repeated to include all available structures for the latter (N = 82). Our shape analysis requires to manually position curves along the cochlea and the three semicircular canals and to define their terminal points. Terminal points are generally placed on homologous structures so as to ensure reproducibility. As we found homologous points difficult to identify confidently, we instead implemented the method described below.

The first Curve (C0) was placed on the external surface of the cochlea starting from the apex of the cochlea (defined as the cupula on the apical turn) and extending to the vestibule (Braga et al., 2015; Coleman and Colbert, 2010). The last point (P1) was placed using the minor axis of an oval window ellipse fit to the cochlea. First, we generated a section at the base of the oval window, i.e. at the junction with the vestibule. The minor axis (S1) of this ellipse-shaped section allowed us to define the point P1 at the intersection with the curve C0. The second curve (C1) was defined by placing a first point at the junction between the lateral semicircular canal and the vestibule while the last point was positioned at the base of the ampulla. The curve was then generated to follow the external surface of the canal. Curve C2 represents the anterior semicircular canal. It was defined as the external curve between a first point located at the base of the corresponding ampulla and a last point that corresponds to the junction of the axes formed by the anterior and posterior canals (P2). The latter also provided the first point for the placement of the posterior canal curves (C3), which extended until the point of intersection (P2, Fig. 2). In the absence of obvious underlying homologous structure, this point (P3) was defined to be at the intersection between a plan (S2, parallel to the anterior canal and tangent to the vestibule) and the axis of the posterior canal.

2.2.3. Statistical analyses

Shape analyses were performed using R studio software (https://rstudio.com/) and consisted of procrustean and principal component analysis as implemented in the geomorph package (Adams et al., 2016; Adams and Otárola-Castillo, 2013). We considered different sets of landmarks for each structure, including 40 landmarks for the cochlea and 20 landmarks for each semi-circular canal (SCC) curve. Linear Discriminant Analysis and predictive scores for both sex and species were produced using a leave-one-out cross validation and confusion matrices. Several metrical characteristics of the bony labyrinth, including the length of the cochlea and each SCC and the number of cochlea turns (Braga et al., 2015), were measured using the MATLAB software (https://fr.mathworks.com/). Non-parametric Kruskal-Wallis and parametric Anova tests were performed using R studio on measurements in order to assess the statistical significance of the differences observed between species among the different structures.

3. Results

3.1. Ancient DNA sex and taxonomic identification

This work builds on the successful sex and taxonomic identification of 102 equine petrosal bones, 58 of which are analysed here for the first-time following sequencing of 191,451–15,381,391 HTS high-quality reads per specimen (Table S1). Ancient DNA authenticity was assessed through the presence of typical patterns of post-mortem DNA decay, including C-to-T (and their reciprocal G-to-A) mis-incorporations at read termini and DNA fragmentation at deaminated Cytosines following USER-treatment (Rohland et al., 2015) (Figure S1). While the vast majority of the specimens were analysed for their DNA content prior to microCT scanning and morphological analyses, nine from Yenikapi were analysed after microCT scanning. They showed similar DNA

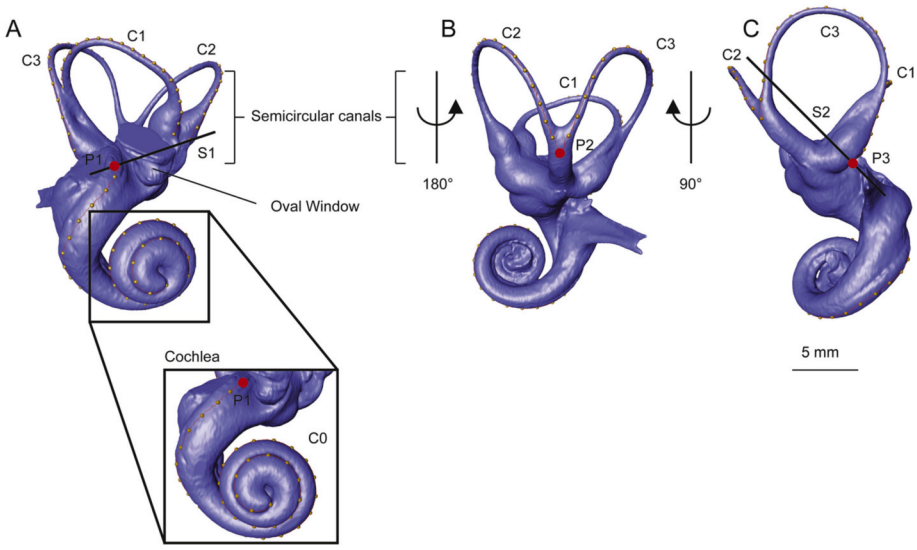

Fig. 2. 3D models from MicroCT scans of the equine bony labyrinth. (**A**) Zoom in the cochlea area, showing the placement of C0 with a terminal point P1 defined by the intersection of the minor axis (S1) of the oval window ellipse and the curve C0. C1 represents the lateral SCC. (**B**) C2 and C3 represent both the anterior and posterior SCCs, which join at the so-called point P2. (**C**) C3 represents the curve tracking the posterior semi-circular canal (SCC) and is defined between the junction of the second and third SCC and point P3. The point P3 is defined as the intersection between a plan (S2, parallel to the anterior canal and tangent to the vestibule, see the tutorial video, Supplementary data) and the curve C3.

preservation levels (Yenikapi site, p-value $= 8.02 \times 10^{-2}$) and sex and taxonomical identification success, indicating that the procedures followed for acquiring 3D models of the inner ear were not significantly detrimental to DNA. Further processing of sequence alignments against the horse nuclear and mitochondrial reference genomes provided sufficient equine reads for both sex and species determination (N $\geq$ 162,902–420,818,091; see Fig. 3 for typical Zonkey assignments). This provided a total of 41 horses (27 males, 14 females), 24 donkeys (20 males, 4 females), 36 mules (16 males, 20 females) and one hinny female for characterization of the inner ear morphology. The limited proportion of hinnies in our dataset is on par with previous reports (Carette 2003; Chaix 2016), indicating anatomical, behavioral and genetic issues limiting production outcomes (Allen and Short, 1997).

3.2. Geometric morphometric (GMM) analysis

We used microCT to generate the 3D models of 102 ancient equine right bony labyrinths for which DNA provided unambiguous sex and taxonomic identification. In order to ensure balanced sampling across all sex and taxonomic categories, DNA analyses were carried out prior to imaging for the vast majority of the specimens (93/102). Ancient DNA analyses are, however, destructive and even careful drilling was found to damage the inner ear morphological structures of 20 specimens (including 6 male horses, 6 horse mares, 2 donkey jacks, 5 male mules and one female mule). This left a total of 82 specimens with both cochlea and SCC data for which the full set of morphological analyses could be carried out. Morphological analyses on the entire panel of 102 specimens were limited to the SCC, in the absence of cochlear information.

3.2.1. Principal component analysis

Shape variation within our data set were analyzed following Procrustes transformation of the landmarks coordinates so as to eliminate possible size covariates. Principal component analyses (PCA) of the cochlear and the SCC shape variables, either taken separately (Fig. 4A and B) or altogether (Fig. 4C), revealed relatively limited overlap between horses and donkeys, suggesting that both species may be successfully discriminated morphologically. However, mules occupied an intermediate position that largely overlapped both parental clusters. Interestingly, the single hinny individual available clustered within the mule morphospace and both hybrids showed larger morphological overlap with the donkey cochlea than that of horses (Fig. 4A), while their SCC appeared closer to those of horses than donkeys (Fig. 4B). This indicates that no clear univocal parental effect dominates the shape of the inner ear structure in equine hybrids. Additionally, we found no morphological separation between individual sexes within the taxonomic groups considered, or between the different countries of provenance of the archaeological material. This demonstrates limited impact of sex and the geographic and temporal origins on the equine inner ear morphological structure.

3.2.2. Linear Discriminant Analysis

In order to assess the predictive power of the structures forming the bone labyrinth, linear discriminant analyses (LDA) were performed on taxonomic groups that were predefined on the basis of the DNA results. LDA were carried out both using the cochlea and SCC coordinates separately, and considering both structures together. Repeating such analyses while excluding those 20 samples for which the SCC structure was damaged during drilling as well as the single hinny specimen did not affect our conclusions (Fig. 5). Cochlea-based LDAs (Fig. 5A, B, E, F) were found to show the lowest predictive scores, limited to 75.0–78.0% for horses, 62.5%–81.0% for donkeys, and 55.6%–64.0% for mules. SCC-based LDAs (Fig. 5C, G) showed improved predictive scores, reaching 83.9% for horses, 85.7% for donkeys and 83.9–87.1% for mules. Finally, considering both structures together improved predictive scores further, reaching equivalent performance for each taxonomic group considered, and ranging between 85.7% and 95.2% scores. Applying similar analyses to sex prediction showed considerably limited performance (data not shown), in agreement with the extensive overlap of male and female morphologies revealed by PCA.

3.2.3. Metrical measurements

In addition to assess differences in the shape of the bony labyrinth amongst horses, donkeys and mules, we explored potential metrical differences between their cochlear length, centroid size and SCC length (Fig. 6). More specifically, we measured the absolute metric length of the cochlea and the relative length (dividing the absolute length by the total number of turns), as well as the length of each individual SCC and the centroid size. The latter was defined as the square root of the sum of squared distances separating all the landmarks of the cochlea and their centroid. We found statistically significant differences between all taxonomic pairs when considering the absolute length (Fig. 6A) and centroid size (Fig. 6C) of the cochlea. Interestingly, while the centroid size of mules was on average intermediate to that of horses and donkeys, their absolute cochlear length the longest. Relative cochlear and SCC lengths were significantly lower in donkeys than in horses and mules. However, these measurements showed no significant differences between horses and mules.

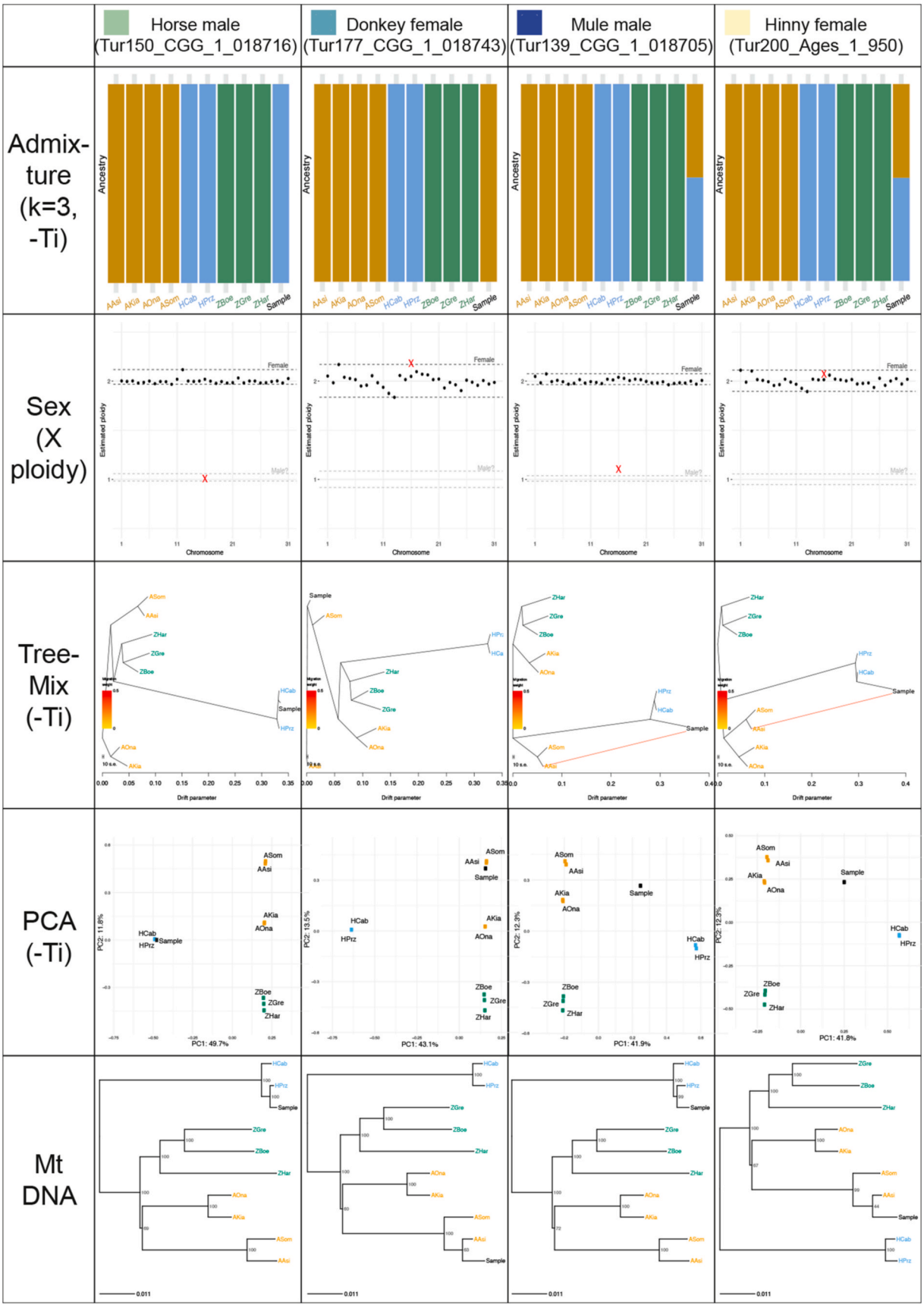

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Fig. 3. Typical Zonkey (Schubert et al., 2017) results obtained on the four categories of equine remains identified. *From left to right:* Specimen Tur150_CGG_1_018,716, identified as a horse male; specimen Tur177_CGG_1_018,743 identified as a donkey female; specimen Tur139_CGG_018,705 identified as a donkey male, and; specimen Tur200_Ages_1_950 identified as the only hinny remain investigated in this study. It belonged to a female individual. *From top to bottom:* the results presented correspond to genome-wide ADMIXTURE (Alexander et al., 2009) ancestry profiles (considering k = 3 genetic components), per-chromosome X ploidy levels for sex determination, TreeMix (Pickrell and Pritchard, 2012) nuclear phylogeny (considering one migration edge in the case of first-generation hybrids), PCA and; RAxML (Stamatakis, 2006) mitochondrial phylogeny. ADMIXTURE, TreeMix and PCA are carried out on autosomal data, excluding transition mutations ("Ti"). Asses: AAsi (*Equus africanus asinus*), Akia (*Equus kiang*), AOna (*Equus hemionus onager*), ASom (*Equus africanus somaliensis*); Horses: Hcab (*Equus caballus*), HPrz (*Equus przewalski*); Zebras: ZBoe (*Equus quagga boehmi*), ZGre (*Equus grevyi*), ZHar (*Equus zebra hartmannae*).

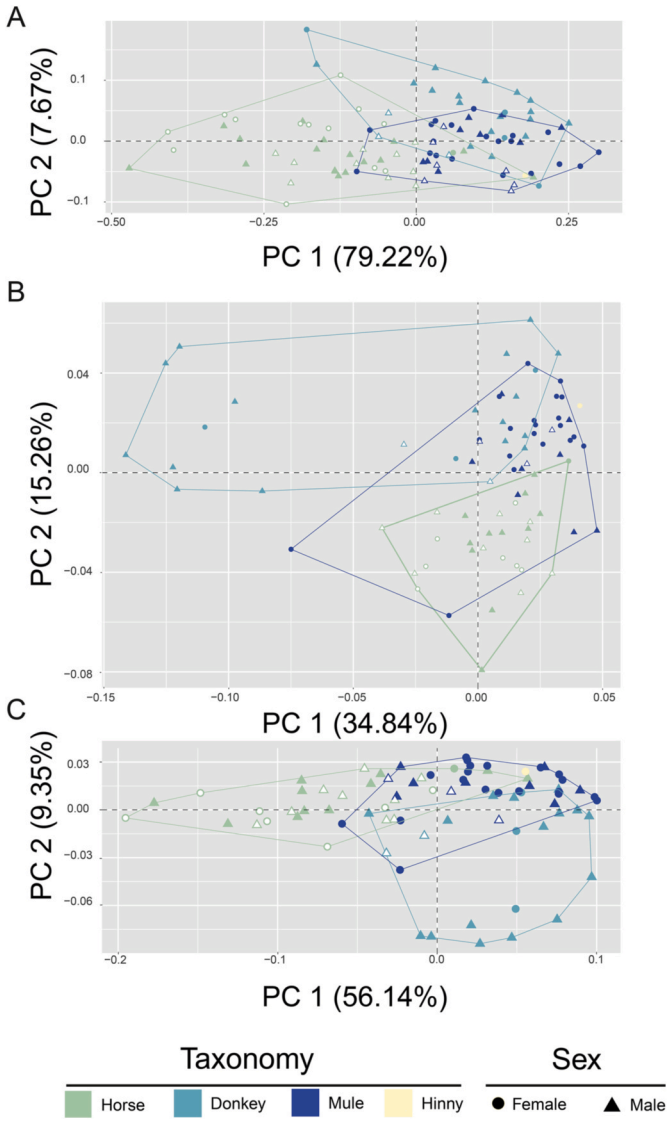

Fig. 4. Principal Component Analyses based on the shape of (**A**) the cochlea, (**B**) the SCCs and (**C**) both structures considered together. Full points indicate specimens from the Yenikapi site from Turkey, empty points indicate specimens from the 10 sites from France. Individual species and hybrids are shown using different colors while molecular sex is reported using triangles for males, and circles for females. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

In this study, we gathered an extensive collection of equine archaeological petrosal bones consisting of 102 specimens from France and Turkey that were spread across the last ~2,000 years. This collection was aimed at encapsulating significant morphological shape variation amongst horses, donkeys and their hybrids. We leveraged a method based on ancient DNA data that was recently developed by some of us (Schubert et al., 2017) to unambiguously determine the molecular sex and taxonomic status of each individual bone material. We then subjected them to a range of morphological analyses in order to assess their respective predictive power for sex and taxonomic determination. Our goal was to mitigate some of the limitations of the methods currently available based on morphological analyses of equine remains that have shown limited performance due to the selection of somewhat debatable classification criteria (Baxter, 1998; Chuang and Bonhomme, 2019; Geigl and Grange, 2012; Granado et al., 2020; Twiss et al., 2017), the limited extent of comparative panels (Albarella et al., 1993; Arbogast et al., 2002; Bökönyi, 1974) and the often-fragmentary nature of archaeological osseous assemblages (Baxter, 1998; Cornette et al., 2015; Owen et al., 2014; Zeder, 1986).

In contrast to previous reports in modern humans (Braga et al., 2019), comparisons of the cochlear shape revealed no significant difference between males and females. This indicates that the predictive power of the labyrinthine morphology for sex determination varies across taxa. Further work is necessary to determine whether the low-predictivity observed amongst equine species represents the rule rather than the exception. Additionally, no differences were detected within species between the different sites and time periods considered (data not shown). This may reflect the limited resolution of the bony labyrinth shape in capturing more subtle, local and/or temporal population structure. Alternatively, this may be due to our study design that may not have sampled the entire range of morphological variation. Future work integrating our data to a more exhaustive geographic and temporal representation, including during the recent history of breed formation, will allow to explore this issue further. However, our work uncovered good-to-excellent predictive power (85.7%–95.2%) for identifying horses, donkeys and mules on the basis of SCC shape variation (Fig. 5). Even though linear SCC measurements indicated that donkeys occupied a smaller size range, they showed limited power for differentiating horses and mules (Fig. 6). Linear cochlear measurements showed instead absolute length and centroid length distributions significantly different amongst all three species pairs investigated, with donkeys characterized with a smaller cochlear length and more compact centroid dispersion. This indicates marked size differences of the bony labyrinth structures across these taxa.

Our study reveals a predictive power for horse and donkey identification that is comparable to that achieved with a recent methodology combining up to 18 cranial and post-cranial bone elements. It, however, outperforms this approach for the identification of hybrids, for which classification success rates of 74.6% were reported (vs 87.1%–93.5% in our study; Hanot et al., 2017). We, thus, conclude that our approach holds unprecedented potential for a reliable assessment of the composition of both horses and donkeys as well as their hybrids in archaeological bone assemblages.

Additionally, our work showed that performing microCT scanning prior to molecular work did not reduce the success of downstream ancient DNA analyses. This mirrors previous reports indicating no significant effects of limited x-ray dose on DNA decay (Hall et al., 2015; Immel et al., 2016). This contrasts to situations in which invasive sampling underlying DNA analyses was performed first, which compromised the acquisition of intact morphological structures in a total of 20 out of 93 specimens (~21.5%), even though particular efforts were made to minimize destruction during drilling. Our work thus suggests that microCT scanning is appropriate to both preserve their morphological

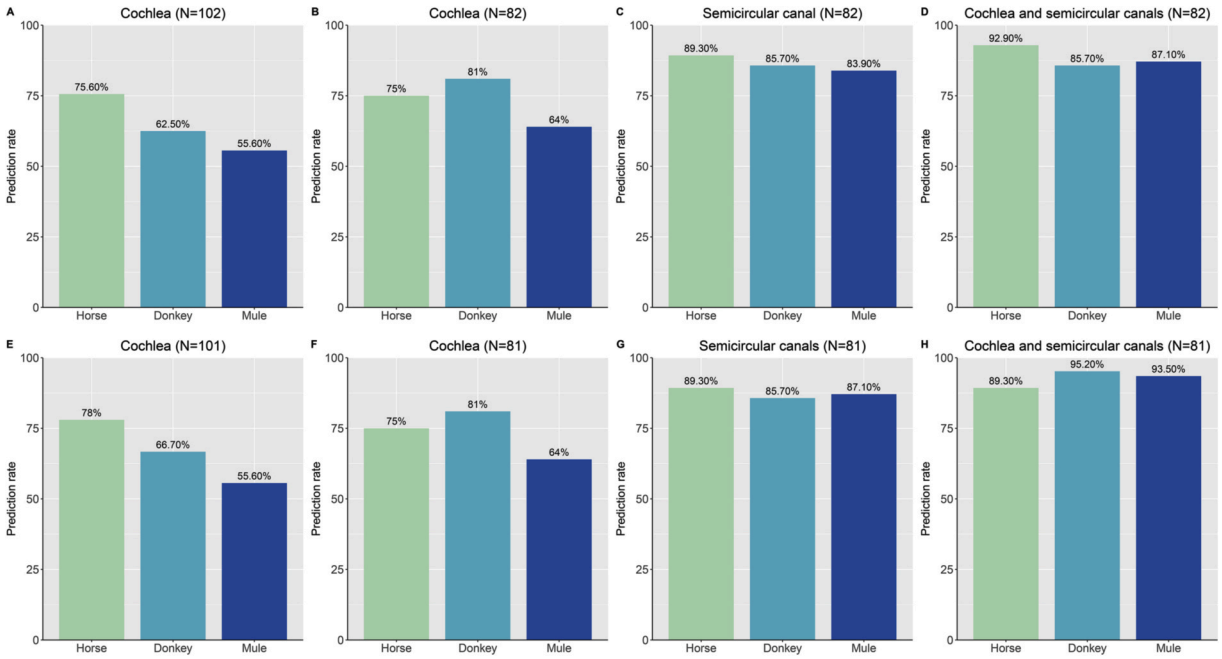

Fig. 5. Linear Discriminant Analysis (predictive rate) based on the cochlea for (**A**) all the specimens (N = 102) or (**B**) removing the 20 with damaged SCC during drilling (N = 82), (**C**) all three SCCs (N = 82), and (**D**) both the cochlea and SCCs (N = 82). Those analyses were repeated, excluding the single hinny specimen available (panels **E** to **H**).

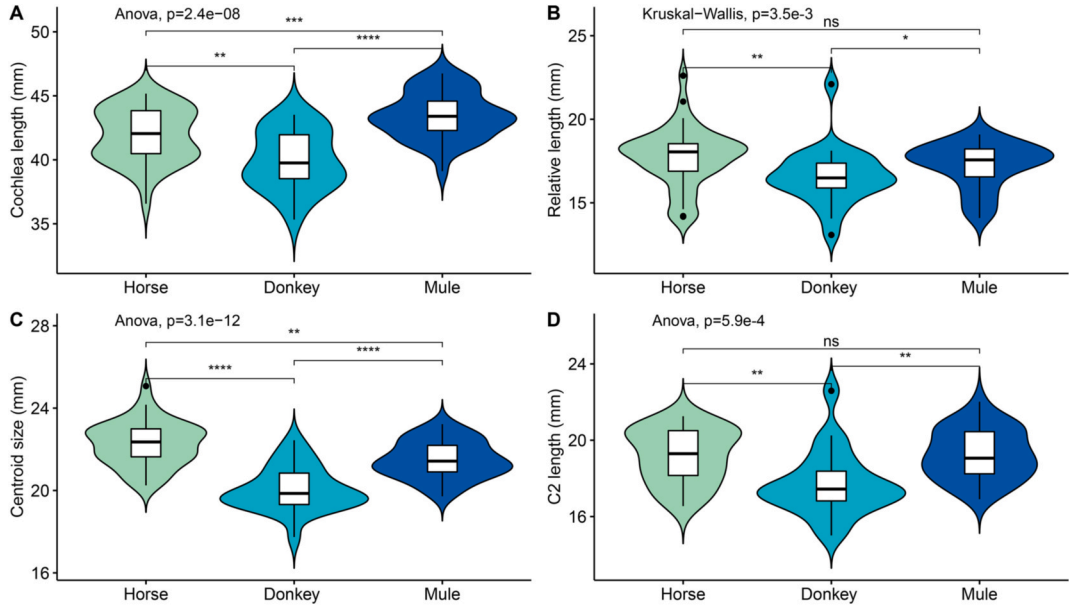

Fig. 6. Distributions of various metrical measurements in horses, donkeys and mules. (**A**) Cochlear length (millimeters, mm). (**B**) Relative cochlea length (defined as the ratio between the total length and the number of turns). (**C**) Centroid size (defined as the square root of the sum of squared distances of all the landmarks of the cochlea from its centroid). (**D**) Anterior SCC length (distributions of the length of the two other SCCs are shown as supplemental Figure S2). The *p*-values of ANOVA and Kruskal-Wallis statistical tests comparing all distribution pairs are provided on top of each plot. Ns: *p*-value > 0.05; *: 0.1 < *p*-value ≤ 0.05; **: 0.001 < p-value ≤ 0.01; ***: 0.0001 < p-value ≤ 0.001; ****: p-value ≤ 0.0001.

information and their full ancient DNA content. Considering the valuable taxonomic information contained and the increasing attractivity of petrosal bones (and related destruction) for ancient DNA analysis, we strongly recommend that microCT scanning procedures similar to those applied here are performed whenever possible and prior to molecular analyses.

Our approach provides a cheap and non-destructive alternative solution to ancient DNA analysis for the identification of horses, donkeys and their hybrids. It can thus be applied to any petrosal bone specimen, regardless of their respective molecular preservation levels. Additionally, our method can be implemented in laboratories not equipped with ancient DNA facilities, assuming local access to microCT instruments. Finally, it is naturally tailored to the study of large bone assemblages as with the instruments used here, only a few hours were necessary to acquire high-resolution 3D models of up to 70 individual samples. Compared with ancient DNA analysis, our method shows, however, several limitations, including the impossibility to discriminate males and females on the one hand, and mules and hinnies on the other hand.

Further work is necessary to assess whether part of these limitations could be overcome using other mathematical frameworks than GMM, for example diffeomorphisms allowing comparisons of complex shapes (e.g. changes in both curvature and torsion along the cochlear length) in a nonlinear geometric framework (Braga et al., 2019). Additionally, mass-spectrometry methods aimed at the characterization of ancient peptides such as ZooMS (Buckley, 2018; Buckley et al., 2014; Stewart et al., 2013) and paleoproteomic analyses (Cappellini et al., 2012; Drake et al., 2020; Maixner et al., 2013) have recently received increasing interest for their taxonomic (Horn et al., 2019), phylogenetic (Buckley et al., 2010; Cappellini et al., 2018) and sex (Froment et al., 2020; Parker et al., 2019; Wasinger et al., 2019) identification potential. Further work may reveal their potential interest in equids, especially for those fragmentary remains other than petrosal that represent the dominant fraction of archaeological faunal assemblages and do not harbor the same DNA preservation levels and morphological internal structures as the petrosal bone.

Data availability

Sequencing data can be found on the European Nucleotide Archive, ENA: PRJEB43564. 3D models are available on MorphoSource (https://www.morphosource.org).

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2021.105383.

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