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# Ancient DNA refines taxonomic classification of Roman equids north of the Alps, elaborated with osteomorphology and geometric morphometrics

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ABSTRACT

Hybrids of horse and donkey, which have been valued in the Mediterranean basin since the Iron Age, became integrated into the animal world north of the Alps in the course of Romanization. Until now, however, their true contribution to the economic and military life in the northern Roman provinces *Raetia*, *Noricum* and *Upper Pannonia* (southern Germany, eastern Switzerland and Austria) has remained unknown in the absence of robust identification methods based on morphometric approaches. We confronted morphological identification of 405 equid specimens collected in the Late Iron Age (Celtic) (~2nd – 1st century (c.) BCE) and Roman (~1st – 5th c. CE) archaeological contexts with their ancient DNA signatures. Our multi-method study demonstrates that although ancient DNA and morphological approaches (including standard osteomorphology and geometric morphometrics) provide overall >85% matching results, in the case of hybrid animals, the extent of overlap with ancient DNA drops to ≤52%. Out of five skeletal elements studied here (mandibular premolars and molars, metapodials, humeri and tibiae), only premolar mandibular teeth ($P_3$, $P_4$) provide good enough accuracy in hybrid classification (89%) based on geometric morphometrics, making it the preferred element and method in future zooarchaeological studies, when ancient DNA data is not available. Moreover, our data show that, although not yet present in the preceding Iron Age, one in six equids in Roman times is a mule, suggesting a strong Mediterranean influence on the use of equids in daily life north of the Alps.

## 1. Introduction

Written records, pictorial evidence and archaeological remains support the presence of different species of equids, including their hybrids across the Roman Empire (Johnstone, 2004; Peters, 1998; Toynbee, 1973). Horses (*Equus caballus*) were much esteemed elite animals, frequently employed in military services and civil life. They were also taking part in Roman amphitheatre games, most prominently in chariot races. Donkeys (*Equus asinus*), to the contrary, were usually exposed to more modest living conditions and represented the

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indispensable pack animals for short and long-distance transportation and trade. However, in rugged mountainous terrain and for carrying heavier loads, mules, *i.e.*, the first-generation offspring between a mare (female horse) and a jack (male donkey), were preferred to donkeys (Armitage and Chapman, 1979; Smith, 2009; Toynbee, 1973). Even though mules are sterile and thus require the maintenance of both parental stocks for breeding, they have been valued until the present day for their strength, stamina, sure-footedness and more balanced temper. Compared to horses, mules can indeed thrive on lower quality food, are more resistant to disease, and even exhibit longer life expectancy (Gollbeck, 1938; Tegetmeier and Sutherland, 1895).

Despite the documented economic and military importance of mules in historic times (Clutton-Brock, 1999; Dent, 1972), their identification in archaeofaunal assemblages with comparative osteomorphology and osteometry is fraught with difficulties (Johnstone, 2004; Peters, 1998; Twiss et al., 2017; Uerpmann and Uerpmann, 1994). This is due to the limited resolution offered by fragmented and/or isolated remains, the paucity of modern reference comparative panels as well as the co-existence of wild and domestic equids and their hybrids over large parts of Eurasia since the Bronze Age. For instance, during the late Bronze Age, western Asia was host to Asiatic wild asses (hemiones), donkeys, horses and their hybrids, *i.e.*, kungas (Asiatic wild ass x donkey) and mules/hinnies (horse x donkey) (Bennett et al., 2017, 2022; Orlando et al., 2006).

Geometric Morphometrics (GMM) (Bignon et al., 2005; Cucchi et al., 2017; Hanot et al., 2017; Seetah et al., 2014; Shackelford et al., 2013) and biomolecular techniques (Schubert et al., 2017) have offered complementary approaches to the identification of equid species and their hybrids. However, the current gold standard for taxonomic classification of equid remains is the Zonkey pipeline, which is based on low-depth high throughput DNA sequencing data (Schubert et al., 2017). It provides maximal sensitivity and specificity even for samples showing poor preservation of ancient DNA (aDNA). Previous studies (Granado et al., 2020; Hite, 2008; Schubert et al., 2017), though dealing with limited sample sizes ($n < 20$), have challenged the robustness of morphological assignments as they do not always recapitulate aDNA results, which has precluded the morphological analyses of extensive series. Even though aDNA analyses have become increasingly popular for elucidating the role of animal taxa in ancient cultures, they imply, however, sufficient molecular preservation, destructive sampling and dedicated infrastructure. Thus, it is essential that novel non-invasive methods leveraging subtle but consistent morphological differences are developed for taxonomic identification. For example, Clavel et al. (2021) have obtained 85.7%–95.2% predictive assignment scores for horses, donkeys and their first-generation hybrids applying GMM to the bony labyrinth (a structure carried within the petrosal bone) 3D shape. However, petrous bones may not always be available in archaeological assemblages, explaining why one has to consider other skeletal elements. That said, the accuracy of morphological approaches such as standard osteomorphology (sMOR) and (GMM) applied to teeth and post-cranial elements, however, remains unknown.

Here we subjected 443 equid specimens collected from eight archaeological sites located in the northern Roman provinces *Raetia*, *Noricum* and *Upper Pannonia* (Fig. 1) to aDNA, sMOR and GMM analyses in order to assess the performance of each method. Whereas the study area was populated by wild horses until domestic horses arrived during the Bronze Age (Librado et al., 2021), mules, and possibly also donkeys, only became popular much later, *i.e.,* once the study area was integrated into the Roman Empire (Deschler-Erb et al., 2002; Peters, 1998; Willms, 1990). Here we note that written agronomic sources like *de re rustica* by Columella (1981) state that mules were much preferred to hinnies, which is why the former is usually meant when discussing Roman

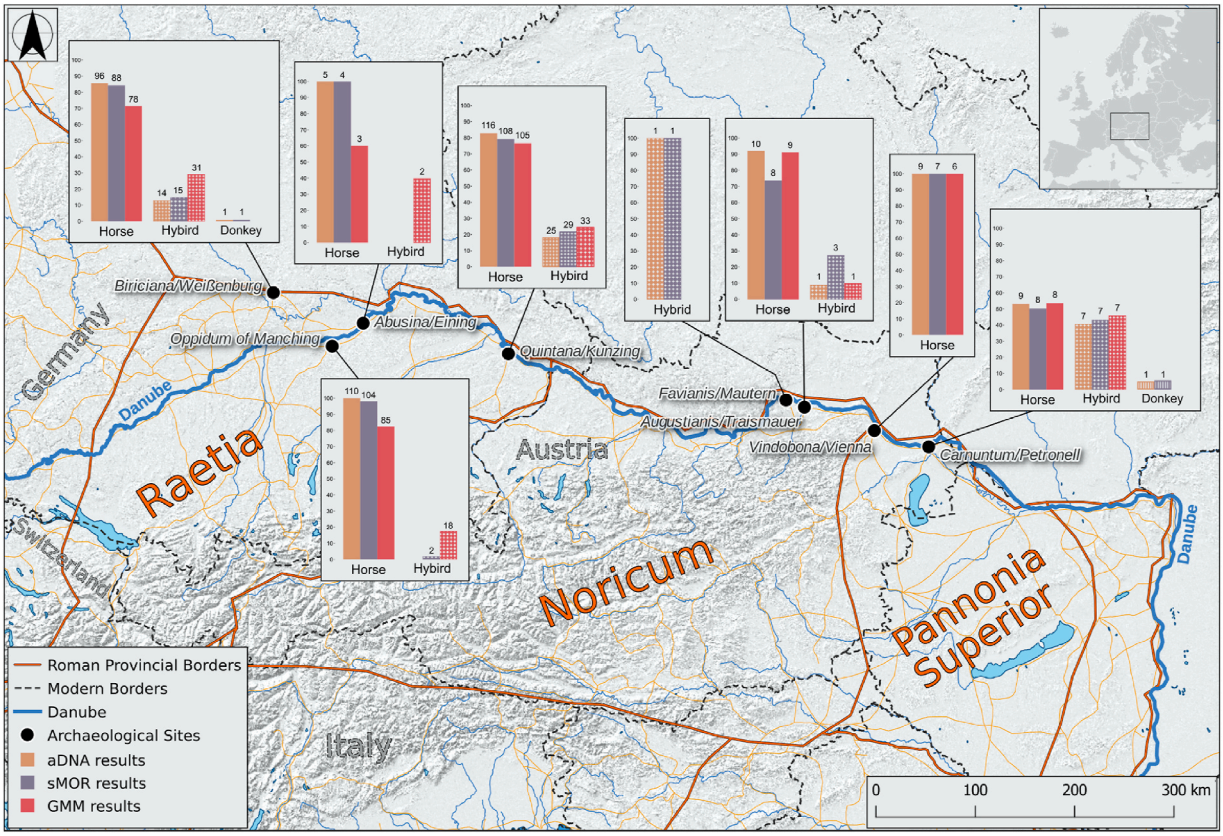


**Fig. 1.** Equid taxa proportions in sites north of the Alps. The bars indicate the proportions of different equid taxa in the provinces *Raetia*, *Noricum* and *Upper Pannonia*. The colors represent the methods (aDNA, sMOR and GMM) and the patterns represent the equid taxa (Horse, Hybrid, Donkey). The numbers on the bars represent the total number of individuals. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

donkey x horse hybrids.

The samples studied by us include 86 lower premolars ($P_3$, $P_4$), 127 lower molars ($M_1$, $M_2$), 102 humeri, 105 metapodials and 94 tibiae. We identify methods and materials allowing robust taxonomic classification of horse, donkey and their first-generation hybrids and provide novel insights into the taxonomic composition of equine herds populating in central Europe during the Late Iron Age and in Roman times. Although limited in time and space, the multi-method approach proposed here can easily be extended to other cultural periods and regions where domestic equids played a prominent role in society.

## 2. Results

Prior to intended analyses, specimens collected in the Late Iron Age site *Manching* (2nd – early 1st c. BCE) and in seven Roman sites located on the *Limes* in *Raetia* (late 1st – 5th c. CE), *Noricum* (2nd – 3rd c. CE) and *Upper Pannonia* (early 3rd c. CE) were morphologically examined by zooarchaeologists experienced in equid osteomorphology. We subsequently applied aDNA, sMOR and GMM analyses to a large subsample likely representing 443 individuals (Fig. 1 and Table 1).

With respect to aDNA, we sampled one skeletal element per individual and generated sufficient low coverage genome-wide data for taxonomic identification of 405 individuals (Supplementary Table S1). The endogenous DNA percentage obtained by us ranged between 0.012% and 60.6% (Supplementary Fig. S1). In the case of morphological analyses (sMOR and GMM), when multiple skeletal elements were available per individual, taxonomic identifications were performed at two levels: *(i)* the skeletal element level, considering each element separately (n = 470), which were subsequently compared to aDNA results to assess the accuracy of taxonomic classification based on respective element, and; *(ii)* the individual level (n = 386), considering all skeletal elements from one individual collectively, which were used to breakdown the proportions of equid taxa in the Late Iron Age and Roman faunal assemblages (Table 1 and Supplementary Table S3, S4).

### 2.1. Late Iron Age and Roman equid assemblages north of the Alps

#### 2.1.1. Ancient DNA (aDNA)

The skeletal elements sampled from 405 individuals comprising 90 humeri, 96 metapodials, 71 tibiae, 147 mandibular and 1 maxilla teeth, provided sufficient aDNA data for taxonomic identification. All the 110 samples from Late Iron Age *Manching* were identified as horses. This contrasted with the Roman assemblage (n = 295), which overall included 245 horses (83.1%), 48 mules (16.3%) and 2 donkeys (0.7%; Fig. 1 and Supplementary Table S1).

#### 2.1.2. Standard morphology (sMOR)

Taxonomic classification using sMOR relies on modern reference collections and distinctive criteria published previously (Baxter, 1998; Dive and Eisenmann, 1991; Eisenmann, 1986; Eisenmann and Beckouche, 1986; Hanot and Bochaton, 2018; Hite, 2008; Johnstone, 2004; Peters, 1998; Taylor et al., 2015) (see Methods). The 386 individuals (470 skeletal elements) that provided sMOR assignments, consisted of 88 humeri, 98 metapodials (94 metacarpals and 4 metatarsals), 74 tibiae, 86 lower premolars ($P_3$, $P_4$) and 124 lower molars ($M_1$, $M_2$) (Figs. 1 and 2b). Morphological diagnosis of mules and hinnies proved impossible for the lack of clear morphological criteria but as already mentioned above, mules were much preferred to hinnies based on Roman agronomist literature. Taxonomic identification based on sMOR confirmed the almost exclusive presence of horses in the *Manching* assemblage (n = 104), except for two individuals classified as hybrids. In the Roman assemblages, sMOR counted 223 horses (79.6%), 55 hybrids (19.6%) and 2 donkeys (0.7%; Fig. 1 and Supplementary Table S3).

**Table 1**
Number of the total and successfully analysed individuals and skeletal elements investigated by three different methods.

| Method | Total individuals | Successfully analysed individuals | Total skeletal elements | Successfully analysed skeletal elements |
|---|---|---|---|---|
| aDNA | 443 | 405 | 443 | 405 |
| sMOR | 405 | 386 | 489 | 470 |
| GMM | 401 | 386 | 485 | 470 |

#### 2.1.3. Geometric morphometrics (GMM)

We first carried out GMM analyses using a modern reference panel comprising all three possible taxa, *i.e.,* horse, donkey and their hybrids. Since we have not identified any donkeys, we removed the donkeys from our modern reference to reduce the risk of erroneous classification and repeated the analyses (Supplementary Table S2). The 386 individuals (470 skeletal elements) that provided GMM classification consisted of 94 humeri, 95 metacarpals, 74 tibiae, 86 lower premolars ($P_3$, $P_4$) and 121 lower molars ($M_1$, $M_2$) (Figs. 1 and 3b). In GMM analyses, our attempt to differentiate mules and hinnies was unsuccessful, hence we report them in a single 'hybrid' category. This is due to the absence of significant shape differences between these two reciprocal hybrids in the reference dataset except in the case of humeri and tibiae (see Supplementary text). The GMM analyses returned a significantly larger proportion of hybrids (n = 18, 17.5%) in *Manching* assemblage than indicated by aDNA and sMOR analyses, while 85 (82.5%) individuals were identified as horses. In the Roman assemblages, GMM analyses found 209 horses (73.9%) and 74 hybrids (26.1%; Fig. 1 and Supplementary Table S4).

### 2.2. Validating the accuracy of sMOR and GMM in equid species identification

#### 2.2.1. Comparison between aDNA and sMOR

Among the 470 skeletal elements, we observed 89.1% (n = 419) agreement in aDNA and sMOR taxonomic classifications and 10.9% (n = 51) conflict (Fig. 2a). Out of 402 horses, 66 hybrids and 2 donkeys identified by sMOR, aDNA results confirmed 384 (95.5%) horses, 33 hybrids (50%) and both donkeys. Chi-square test revealed significant difference in the correct identification of horses vs hybrids ($p < 0.01$, Supplementary Table S6). Among the 33 skeletal elements where genetically identified horses showed hybrid-like morphology, the most prominent element appeared to be the humeri (36%), but other elements also contributed (tibiae: 21%, metapodials: 18%, molars: 12% and premolars: 12%). Conversely, among the 18 skeletal elements misidentified as horse by sMOR, the most common element was molars (39%), followed by metapodials (28%), humeri (16.7%), tibiae (11%) and premolars (5.6%, Fig. 2a).

Considering simulation data showing that Zonkey provides almost 100% specificity with the amount of aDNA data generated (Schubert et al., 2017), the overall performance of morphological approaches can be assessed respective to aDNA-based assignments. The overall consistency rate of sMOR assignments was equal to 89.1%, considering all dental and skeletal material collectively. Amongst the different skeletal elements studied, premolars were found to exhibit the highest rate of accuracy (94.2%), followed by molars (91.1%), metapodials (88.8%) and tibiae (87.8%). Humeri showed significantly lower performance (83%, $p = 0.038$ and $< 0.05$, Fig. 2c and Supplementary Table S6).

Interestingly, morphological misidentification was not exclusively limited to fragmented bone elements but also affected complete mandibles (n = 57), and occasionally even complete skeletons (n = 5). In fact, two out of 57 complete mandibles carrying both $P_3$ and $M_2$ provided incorrect sMOR assignment, while in two other mandibles, the two teeth examined returned contradictory results (Supplementary Table S3). In the case of complete skeletons, our analyses included seven elements, i.e. $P_3$, $P_4$, $M_1$, $M_2$, humerus, metacarpal and tibia. Two of the five complete skeletons returned wrong assignments for either one (QUI-53-1, metacarpal) or two elements (QUI-55-1, humerus and tibia, Supplementary Table S3).

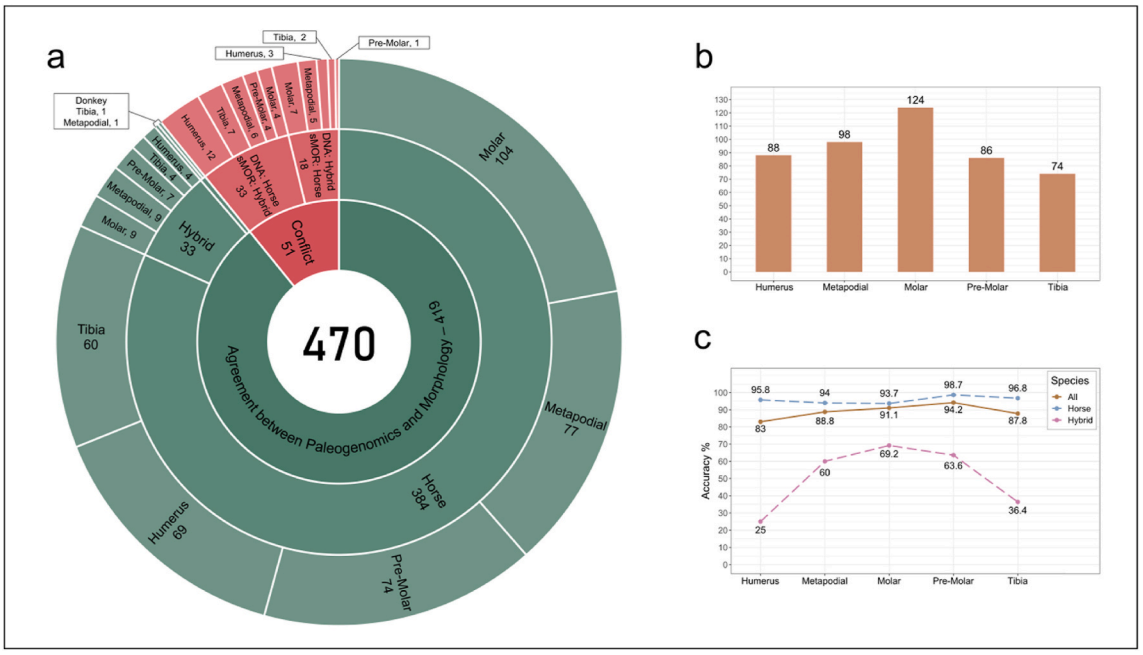


**Fig. 2.** Comparison between ancient DNA and standard morphology. (a) The inner most circle (white) shows the total number of skeletal elements examined. The inner pie chart shows the overall agreement (green) and conflict (red) in species identification between sMOR and aDNA. The middle and outer pie charts represent the number of different species and the number of different skeletal elements in each category, respectively. **(b)** The number of cranial and post-cranial elements provided species assignments using both sMOR and aDNA analyses. **(c)** Accuracy of sMOR analyses in different skeletal elements, assessed using aDNA results. The category "All" represents parental species and their hybrids. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

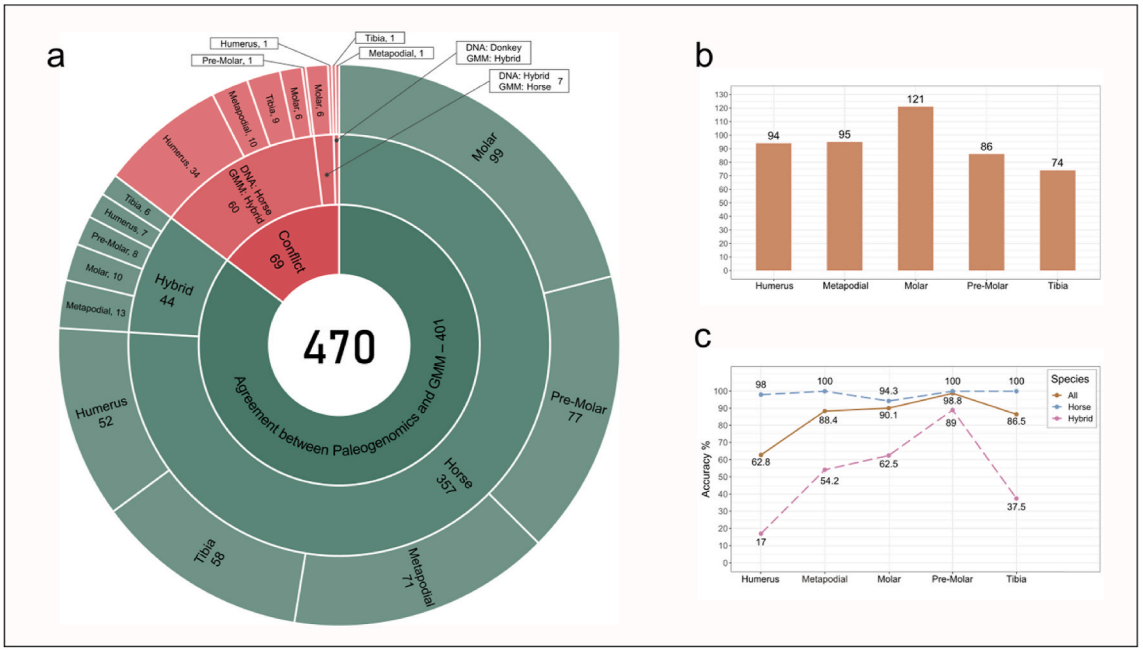


**Fig. 3.** Comparison between ancient DNA and geomorphic morphometric. (a) The inner most circle (white) shows the total number of skeletal elements examined. The inner pie chart shows the overall agreement (green) and conflict (red) in species identification between GMM and aDNA. The middle and outer pie charts represent the number of different species and the number of different skeletal elements in each category, respectively. **(b)** The number of cranial and post-cranial elements provided species assignments using both GMM and aDNA analyses. **(c)** Accuracy of GMM analyses in different skeletal elements, assessed using aDNA results. The category "All" represents parental species and their hybrids. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### 2.2.2. Comparison between aDNA and GMM

Among the 470 skeletal elements, we observed 85.3% (n = 401) agreement in aDNA and GMM taxonomic classifications and 14.7% (n = 69) conflict (Fig. 3a). Out of 364 horses and 106 hybrids classified by GMM, aDNA results confirmed 357 (98.1%) horses, and 44 hybrids (41.5%). In the conflicting category, in which genetically identified

horses or donkeys were misclassified as hybrids by GMM (n = 62), the most common element was humeri (56.7%), followed by metacarpals (16.7%), tibiae (15%), molars (10%) and premolars (1.7%). Conversely, among the seven elements misidentified as horses by GMM, the major contribution was by molars (85.7%), followed by humeri (14.3%, Fig. 3a). In line with sMOR results, premolars exhibited the highest accuracy (98.8%; $p < 0.001$), closely followed by molars (90.1%), metapodials (88.4%), and tibia (86.5%). Humeri, however, again showed the poorest performance (62.8%; $p < 0.001$) (Fig. 3c, Supplementary Table S7).

Like sMOR and on the basis of the same subsample, we observed cases of species misidentification in complete mandibles and skeletons. In fact, only one of the five complete skeletons (QUI-56-1) showed correct GMM taxonomic assignment for each element. In all remaining cases, one (either humerus or $P_3$) or occasionally even two elements ($M_1$ and tibia) had been misclassified. Additionally, we observed that of a total of 57 complete mandibles with all premolars and molars in situ, four of them with both $P_3$ and $M_2$ preserved returned incorrect GMM assignments for the latter tooth (Supplementary Table S4).

## 3. Discussion

### 3.1. Morphological identification methods: Room for improvement

In this study, we applied multiple classification methods to identify the taxonomic composition of the Late Iron Age and Roman equid assemblages collected in sites located north of the Alps. Our approach reveals variable morphological mis-classification rates across skeletal elements and methods. Our results seem not in line with expectations of GMM performing better than sMOR. This surprising result most likely reflects limitations of the collection of modern reference specimens forming our comparative baseline, showing both insufficient numbers of remains, and a morphological range not on par today and in the past. We tried to overcome such limitations by including modern horses, donkeys and hybrids from different European natural history collections, but except for horses, the other taxa and hybrids in particular are only presented in small numbers. Moreover, modern breeding has considerably affected the horse morpho-anatomy, and animals of small stature (≤130 cm at the withers) remain relatively rare. The absence of normal-proportioned small stature horses in our reference panel probably inflated the proportion of misclassifications into other categories where animals are generally of smaller stature (albeit exceptionally large donkeys have also been documented in Roman assemblages from France (Lepetz et al., 2021)).

Due to the sensitivity of the linear discriminant analysis (LDA) to large differences in sample size between reference taxa, we homogenized the sample size between horses and hybrids by removing the outliers. These modern outliers include ponies, draft horses and some lineages comprising mixed breeds. As the overall shape of the Iron Age horses differs from their Roman and modern relatives, in particular regarding the size proportions of different cranial to postcranial elements, by removing the outliers we misidentified a large proportion of these small horses as hybrids (Supplementary Fig. S2 and Text). Understandably, additional modern reference specimens would help optimize taxonomic classification based on shape. However, enlarging modern reference collections is expensive and requires adequate facilities. While horses appear unproblematic, the acquisition of hybrids is more complicated for ethical reasons. In sum, the identification of hybrids applying GMM will remain challenging as long as the foundation for comparison cannot be adequately increased. The bias characterizing our modern baseline can help explain why 16.8% of the small-sized equids of Celtic *Manching* were wrongly classified as hybrids by GMM.

Our results show that amongst the different skeletal elements analysed by sMOR and GMM, lower premolars show the highest overall accuracy in the taxonomic classification of equids, in line with previous work (Clavel et al., 2021; Cucchi et al., 2017; Hanot et al., 2017), followed by molars, metapodials, tibiae and humeri, which show particularly poor performance, especially in hybrids (Fig. 4). This is likely due to the often-fragmentary nature of the bone, limiting the analyses to the early fusing distal articular end, which was previously reported to result in lower accuracy (Hanot et al., 2017). Accordingly, removing humeri improved GMM performance, reducing misclassification rates from 14.7% to 9%. Interestingly, contrary to the expectation that the two morphological approaches applied by us would harmonize in terms of disagreement from aDNA results, we noted that only nine out of 110 specimens fit this assumption (Supplementary Fig. S3 and Text). It should be mentioned that in morphological classification of mandibular teeth, we combined classical morphology with morphological criteria resulting from Generalized Procrustes Analysis (GPA) (see Methods). It is therefore possible that the latter enhanced the rate of correct identification of premolars and molars in sMOR.

Although genetic evidence for hinnies is lacking in the archaeological dataset, GMM analyses of humerus and tibia identified 12 hinnies in the Roman assemblages from *Biriciana*/Weißenburg and *Quintana*/Künzing (Table S4). Despite significant shape differences in humerus and tibia between mules and hinnies, we could not capture the variation in our dataset due to the underrepresentation of these hybrids in the modern reference collection, which affects the correct shape description in LDA analysis. Arguably, the discrimination rate between mules and hinnies will improve in case hybrid sample size can be increased.

In the light of our results, future work should mostly focus on increasing the size and diversity of the reference panels used in GMM analyses. This can be effectively achieved by running aDNA analyses on a vast collection of equid remains (as done in this study and in Lepetz et al. (2021)) so as to identify and increase the representativity of hybrids in the morphological reference panels. This strategy was proved successful in Clavel et al. (2021), in which the authors obtained 85.7%–95.2% taxonomic classification rates in horses, mules and donkeys applying GMM analyses to the bony labyrinth of the petrous bone. Considering most of the bones in faunal assemblages are postcranial elements, using our aDNA dataset which consists of various skeletal elements would be the next step to improve the reference panel for GMM analyses.

### 3.2. Equid exploitation north of the Alps from the Late Iron Age until Roman period

From an historical perspective, investigation of the conspicuous equid assemblage from the Celtic *oppidum* of *Manching* formed a meaningful starting point for our study. Its faunal assemblage consisting of more than 450,000 remains of domestic mammals and roughly 22,500 of domestic horses (5%) provides a statistically reliable sample size representing the population (Boessneck et al., 1971; Obermaier, 2013). In addition, probably the largest proto-urban settlement north of the Alps, *Manching* was involved in long-distance trade with the Mediterranean region, reflected by the consumption of wine and fermented fish sauce (garum, liquamen) (Manhart, 1998; Sievers, 2003). As such, earlier morphological work did not come across mules in pre-Roman Iron Age contexts north of the Alps (Boessneck et al., 1971; Manhart, 1998; Peters, 1998; Trixl, 2019), but some doubts remained regarding a possible late Iron Age presence of hybrids north of the Alps. This is due to the discovery of the oldest mule identified in Saint-Just (France) belong to the terminal *La Tène* period (late 1st c. BCE and the early 1st c. CE) in Saint-Just (France) (Fages et al., 2019).

Ancient DNA could not confirm the presence of mules in the pre-Roman northern Alpine foothills either, thus substantiating earlier conclusions that only horses served as mounts and pack animals in the Late Iron Age. A parallel finding was recently made by Lepetz et al. (2021) after evaluating >130 equids from *Hallstatt-La Tène* (7th – 1st c. BCE) contexts across France. Based on the foregoing information, researchers in charge of archaeofaunal collections may appreciate to know that in the regions of study, exploitation of hybrids seem to have been

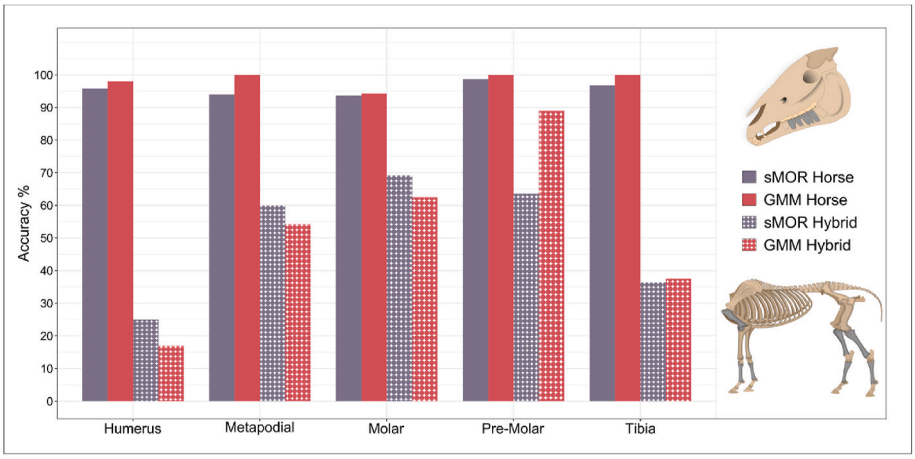


**Fig. 4.** Comparison between geometric morphometrics and standard morphometry. The accuracy of various skeletal elements in equid taxa (horse *vs.* hybrid) identification, applying GMM and sMOR. The colors represent the methods (sMOR and GMM) and the patterns represent the equid taxa (Horse and Hybrid). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the absolute exception prior to the Roman era. From a conservation point of view, invasive sampling to detect hybrids in Iron Age or earlier contexts in France and southern Germany thus becomes almost superfluous.

Turning to the Roman assemblages, aDNA reports on the prevalence of 83.1% horses, 16.3% mules and 0.7% donkeys (245:48:2), confirming the value of mules alongside horses for army operations as described in the literature and highlighted in Roman historic sources (Peters, 1998; Rost and Wilbers-Rost, 1993; Toynbee, 1973; Uerpmann and Uerpmann, 1994). The cultural pattern observed north of the Alps corresponds well to the findings from Roman France, where mules contributed 20.0% and 34.2% of the equine assemblages between the end of the 1st and the beginning of the 3rd c. CE, respectively (Lepetz et al., 2021). Despite being based on a much smaller sample size (n = 19), a similar pattern with a dominance of horses (46%) and a secondary role for mules (16%) was reached applying aDNA analyses to a Roman equine assemblage from Switzerland (Granado et al., 2020). Regardless of the location and the number of samples, all these studies conclude that horses clearly outnumbered mules in Roman deposits.

Finally, if we consider aDNA as the golden standard and the Zonkey pipeline ideal for tracing hybrids in archaeofaunal assemblages (Schubert et al., 2017), it can now be safely assumed that in our study area, on average one-sixth of the equid population exploited in the study area were mules. However, we also acknowledge the regional differences as in the Roman assemblages from France, on average 1 out of 5 equine remains were identified as mules. From our aDNA results and compared to Lepetz et al. (2021), however, donkeys were surprisingly few in numbers in the archaeological contexts investigated by us. Earlier work (Willms, 1990) assumed a higher density of donkeys in southern Germany, but on closer examination of the specimens and/or the archaeological contexts from which they originated, fundamental reservations arose, especially with regard to their dating. Because of medieval settlement layers overlying Roman occupation, the presence of intrusive specimens could not be entirely ruled out (Peters, 1998). Whereas aDNA analysis combined with direct radiocarbon dating will improve our knowledge regarding the whereabouts of donkeys north of the Alps, the fact that mule breeding does not require large donkey stocks could be used as an argument in favour of local efforts to breed these hybrids.

However, in contrast to the study conducted in France (Lepetz et al., 2021), wherein the large majority of samples originated from geographic regions principally enjoying oceanic or semi-oceanic weather conditions and thus on average comparably mild climates (https://about-france.com/climate-weather.htm), the situation is different in southern Germany and northern Austria, characterized by a continental climate with cold winters. Thus, from a climatic viewpoint, the Roman provinces of *Raetia* and northern *Noricum* were clearly less suitable to thermophilic donkeys compared to large parts of *Gallia*, challenging the idea of continuous breeding efforts north of the Alps. In this respect, the few stable isotope signatures available for Roman mules confirm an early life stage characterized by warm conditions typical of a Mediterranean location and a subsequent transfer to a significantly cooler environment (Berger et al., 2008), in some cases explicitly in the context of Roman campaigns to conquer the northern alpine forelands and regions further north (Uerpmann et al., 2007; Uerpmann and Uerpmann, 1994). Based on the findings by Lepetz et al. (2021), however, the possibility that *Raetia* was stocked with mules bred in *Gallia* must now be considered as well. Having said that, climatic, archaeological and geochemical evidence nonetheless point to the Mediterranean basin as a source of mules for Roman military operations north of the main Alpine ridge.

## 4. Conclusion

The methodical triad applied in this study, including sMOR, GMM and aDNA analyses, provides unique insights into the respective performance and limitations of each. Although all methods confirm the wide presence of mules in Roman *Raetia*, *Noricum* and *Upper Pannonia*, results of taxonomic classification vary considerably between the different skeletal elements investigated by us. Here we demonstrate that the lower premolars ($P_3$, $P_4$) maximize the chances to detect mules in the archaeological record and should be preferred for those laboratories not equipped with dedicated aDNA facilities and/or in cases where invasive sampling is not an option. Additionally, our study clearly illustrates the limitations of the modern reference collection for tracking past patterns of mule exploitation in temperate Europe based on morphology and shape analysis. This can be improved by applying the approach proposed in Clavel et al. (2021) using the most prevalent dental and postcranial remains. For now, and until a more comprehensive reference collection can be made available, taxonomic classification of equine assemblages should ideally be confirmed using palaeogenetic approaches including the Zonkey pipeline (Schubert et al., 2017).

The aDNA results presented in this study revealed a major shift in composition of equid herds initiated with the Roman conquest, when for the first time mules and occasionally even a donkey can be evidenced north of the Alps. Particularly the breeding of mules must have been a lucrative economic activity, considering that in the Roman assemblages studied by us, every sixth equid was a mule. With a ratio horse to mule of 4 to 1 and clear evidence for male donkeys, hybridization was practiced

locally in *Gaul* (Lepetz et al., 2021). Further east in continental central Europe, however, such efforts may have been thwarted due to the climatic conditions specific to our study area. In this respect, geochemical analyses of mules exploited in Roman military service illustrate that the animals first grew up in warmer regions before they were brought over the Alps, which confirms that hybridization had taken place in breeding centers south of the Alpine divide (Berger et al., 2008). It remains to be seen, however, whether this stayed the only option to the military once the army units became stationary and controlling the borders towards the so far unoccupied territories of Central Europe became their main task. Future research will thus have to deal with the question of whether there were local breeding efforts or even, at times, hybrid imports from *Gaul* or from other parts of the Roman Empire.

## 5. Methods

### 5.1. Sample collection

We analysed modern equid skeletons as well as the Late Iron Age and Roman equid remains collected in archaeological sites in the Roman provinces of *Raetia*, *Noricum*, and *Upper Pannonia*. While modern samples were exclusively used as the baseline for morphological comparison, archaeological specimens were investigated both morphologically (sMOR and GMM) and genetically (aDNA).

We applied the following strategy to minimize multiple sampling in aDNA analysis. In the presence of the complete skeleton, one skeletal element per animal was sampled, preferably a lower mandibular tooth, due to greater likelihood of DNA preservation (Hansen et al., 2017). However, in the absence of a complete skeleton, we restricted the sampling to lower mandibles (teeth in-situ), as well as a certain body side (right *vs.* left) for the various postcranial (humerus, metapodials, tibia) elements per archaeological site. Here, we refer to these samples as "individuals", assuming that we selected one skeletal element per animal. Apart from the individuals that were completely recovered during excavation, the faunal specimens analysed here represent the waste products of consumed slaughtered horses as well as carcasses that were disposed in ditches and open spaces, with bone specimens distributed across larger areas and in different parts of the anthropogenic areas. The likelihood that an individual animal was sampled twice is therefore low. In contrast, different skeletal elements were selected for each individual (referred as "skeletal elements") to assess their respective power for species identification on the basis of sMOR- and GMM-based analyses. The description and location of the archaeological sites including the number of samples per site can be found in the Supplementary Text and Fig. 5, respectively.

#### 5.1.1. Modern equids (horse, mule, hinny)

To investigate the shifts in equine taxonomic composition and exploitation in Roman times, we generated a modern baseline comprising 117 modern individuals including 85 horses, 23 mules and 9 hinnies housed at various Museums and Institutions (Supplementary Table S2). Not all modern equids investigated were complete individuals, as in many cases only skulls were available. In the end, the baseline available for comparison summed up to 335 skeletal elements and included 201 mandibular teeth, 41 humeri, 52 metacarpals and 41 tibiae. Due to the small sample size of hybrids (mules and hinnies) in the modern reference collection, no significant differences in shape were observed, except for humerus and tibia. We, thus, decided to pool them together in a single group of first-generation hybrids (Supplementary Table S2).

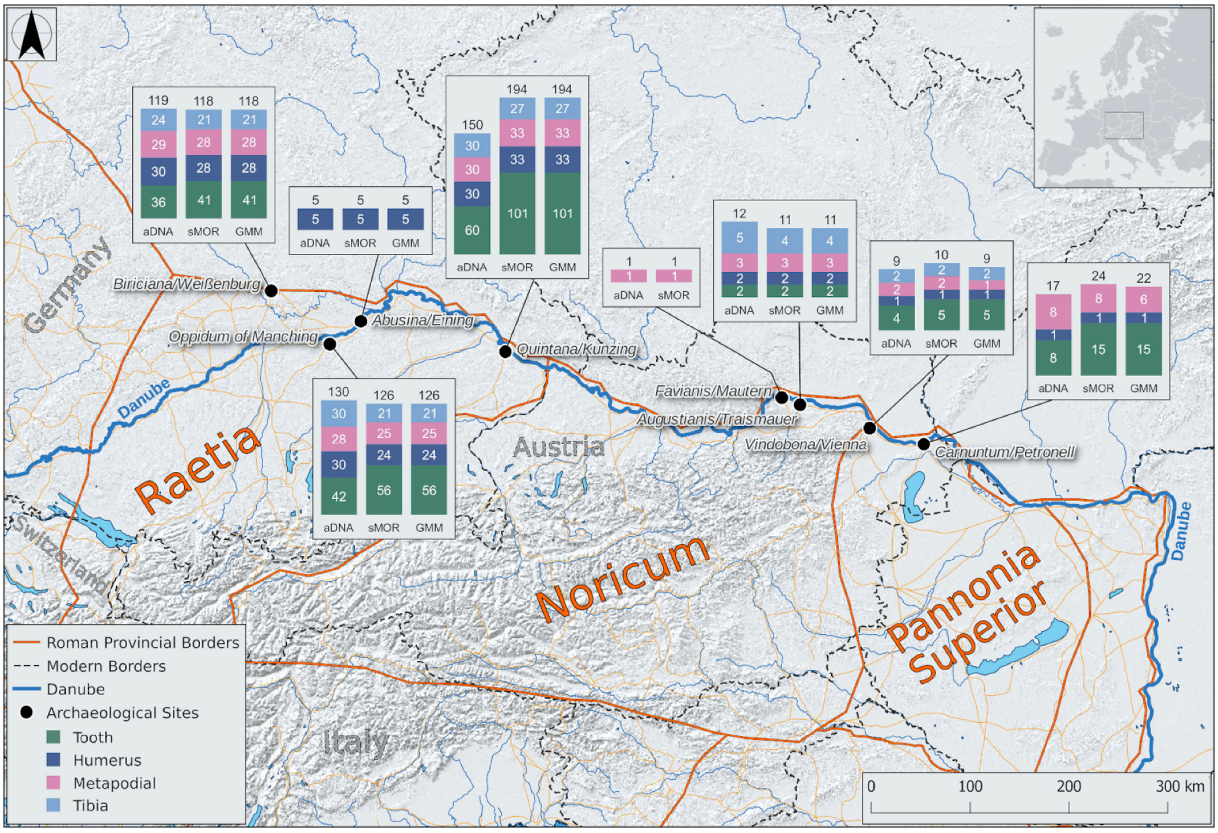


**Fig. 5.** The location of the archaeological sites along the Limes *Germanicus*, *Norici* and *Pannonicus*, North of the Alps. The barplots indicates the number of various cranial and postcranial skeletal elements from the Late Iron Age and Roman sites analysed in this study. The colors represent the different skeletal elements. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

#### 5.1.2. The Late Iron Age equids

In order to morphologically and genetically characterize the equid populations present in the northern Alpine forelands prior to the Roman conquest, we selected 130 individuals from the Celtic *Oppidum* of *Manching*. This site is one of the largest proto-urban settlements north of the Alps, with equid remains presumably representing the local *La Tène* horse lineages bred from the late 3rd until the early 1st c. BCE (Boessneck et al., 1971). The huge faunal collection available for this site is curated at Bavarian State Collection of Anthropology and Palaeoanatomy, Munich (SNSB) (Fig. 5, Supplementary Table S1).

#### 5.1.3. The roman equids

A total number of 313 individuals were selected from various Roman sites along the Limes in *Raetia*, *Noricum* and *Upper Pannonia*. In *Raetia*, a total of 274 individuals were selected, including 119 from *Biriciana*/Weißenburg (Peters, 1998; Sachenbacher-Palvestra, 1991), 150 from *Quintana*/Künzing (von den Driesch and Cartajena, 2001), and 5 from *Abusina*/Eining (Lipper, 1981) (Fig. 5, Supplementary Table S1). A total of 13 individuals were selected from *Noricum*, which includes 12 individuals from *Augustianis*/Traismauer (Riedel, 1991) and a single individual from *Favianis*/Mautern (Kunst, 2006). Finally, a total of 26 individuals were selected from *Upper Pannonia*, including 17 from *Carnuntum*/Petronell (Kunst, 1997, 2000) and nine from *Vindobona*/Vienna (Czeika, 2001) (Fig. 5, Supplementary Table S1). A detail description of the Roman archaeological sites is available in the Supplementary Text.

### 5.2. Sample preparation and DNA extraction

All samples were processed at the state-of-the-art aDNA facility at the Chair of Palaeoanatomy, Domestication Research and the History of Veterinary Medicine, LMU (Munich, Germany), following standard contamination precautions (Knapp et al., 2012). To remove surface contamination, the samples were firstly UV-irradiated for ~30 min and then mechanically cleaned with low speed Dremel using a "7105 diamond wheel point" bit. After cleaning, 50–100 mg of bone powder was collected using a cleaned bit and submitted to DNA extraction following the method described in Dabney et al. (2013). Extraction blanks were included in each extraction batch (n = 45) to monitor any cross-contamination during our experimental workflow.

### 5.3. Library preparation and shotgun sequencing

Illumina double-stranded DNA libraries (DSLs) were built directly from the DNA extracts as well as extraction blanks and negative controls (library blanks), following Meyer and Kircher (2010) protocol using double indexed adapters. The purified and indexed libraries were first analysed on Agilent Bioanalyzer DNA high sensitivity or DNA 1000 kit, quantified using Qubit high sensitivity dsDNA kit and then pooled in equimolar concentration, prior to low-depth shotgun sequencing (0.6–30 million pair end "PE" reads per sample) on Illumina platforms (150 PE, HiSeq X-10 and Novaseq 6000 S4).

### 5.4. aDNA data analyses

#### 5.4.1. Read processing

We used the open-source PALEOMIX v.1.2.14 software (Schubert et al., 2014) which automates read trimming, collapsing of overlapping mate-pairs, read mapping and PCR duplicate removal. More specifically, raw reads were trimmed for adapter/index sequences and low-quality bases at end (min quality 25) using AdapterRemoval v2.3.1 (Schubert et al., 2016), and collapsed when mate-pairs were available and overlapped significantly (-mm 3, 11 bp). The non-collapsing mate-pairs were discarded as likely including a significant fraction of contaminants. Collapsed and collapsed-truncated reads were further filtered for a minimal length of 30 bp, before being aligned against the horse (*E. caballus*) nuclear (EquCab2: GCA_000002305.1) and mitochondrial (NC_001640.1) reference genomes using BWA v.0.7.16a (Li and Durbin, 2009). We disabled seed option for mapping and followed the recommended parameters (-n 0.02 -o 2 -i 0 -q 0) from Schubert et al. (2012). Alignments with mapping quality <25 were discarded and PCR duplicates were removed using MarkDuplicates program from Picard tools (http://www.picard.sourceforge.net). Finally, read alignments were realigned around indels using GATK indel realigner v3.8.1.0 (McKenna et al., 2010).

#### 5.4.2. aDNA authentication

DNA degradation and the damage pattern authenticating aDNA were examined from 100,000 randomly selected mapped reads using mapDamage 2.0 (Jónsson et al., 2013), with default parameters. Nucleotide misincorporation patterns, consisting of elevated C to T substitutions towards sequencing starts (and complementary inflated G to A substitution rates towards sequencing ends), as well as purine (pyrimidine) enrichment of genomic positions preceding read starts (ends) were considered as indicative of aDNA (Briggs et al., 2007; Sawyer et al., 2012). The individual bam files were also rescaled (–rescale) in order to lower the quality scores and, thus, the impact of damaged bases in downstream analyses.

#### 5.4.3. Identification of equids and F1-hybrids

The rapid and robust identification of equine species and their F1-hybrids was carried out using the open-source Zonkey pipeline (Schubert et al., 2017) implemented in PALEOMIX v.1.2.14 (Schubert et al., 2014). A minimum number of 1000 SNPs (resulting from ~2000 high quality mapped reads) were considered sufficient for accurate classification as recommended in Schubert et al. (2017). Zonkey contains a diagnostic panel of 36.5 million SNPs built from nine equine genomes (two caballine and seven non-caballine). The identification of equid taxa and sex of the specimens is based on RaxML (Stamatakis, 2014) mitochondrial and TreeMix (Pickrell and Pritchard, 2012) nuclear phylogeny, ADMIXTURE (Alexander et al., 2009) ancestry profiling, principal component analysis (PCA), and the relative coverage of autosomal and X-chromosomal DNA. Typical Zonkey results for each equid taxa identified here are shown in the Supplementary Fig. S4.

### 5.5. 2D GMM analyses of dental and 3D GMM of postcranial remains

Mandibular cheek teeth $P_3$ and $M_2$ were subjected to GMM analyses due to their accurate separation from neighboring $P_4$ and $M_1$ based on the application of predictive discriminant analysis of GMM, performed in Cucchi et al. (2017). However, in case of complete skeletons, comparisons were extended to four mandibular cheek teeth ($P_3$, $P_4$, $M_1$, $M_2$) and postcranial elements. The 2D GMM protocol was performed on mandibular cheek teeth following Cucchi et al. (2017) which was tailor-made to quantify previously described morphoscopic criteria for identifying equid taxa (Eisenmann, 1986). The occlusal enamel patterns of the teeth were digitized on the pictures taken by a Canon 70D and Canon EF-S 60 mm f/2.8 Macro USM lens and the ancient pictures taken by Véra Eisenmann (available at www.vera-eisenmann.com and in Cucchi et al. (2017)), using TpsDig2 version 2.17 (Rohlf, 2010). The 3D GMM analyses was carried out on complete and in case of fracture, on the distal parts of humerus, metacarpal and tibia. Following Hanot et al. (2017), we evaluated 47, 31 and 41 landmarks (20, 13 and 15 for distal) on humerus, metacarpal and tibia, respectively. The 3D coordinates of the landmarks were registered using a Microscribe 3D digitizer. In principle, the left teeth and bones were digitized when available and in case of absence, the right teeth and/or bones were mirrored to left.

GPA was performed on 2D and 3D points coordinates to remove the effects of position, size and orientation of bones and to put all individuals at the same morphological space (Bookstein, 1991; Cucchi et al., 2015; Rohlf and Slice, 1990); and produce both shape (Procrustes residuals) and size variables (centroid size). PCA analyses were carried out using the Procrustes residuals to reduce the dimensionality of the

shape dataset (Baylac and Frieß, 2005). To compare shape differences amongst different taxa, a Multivariate analysis of variance (MANOVA) with pairwise permutation test (n = 999) was used. Differences in size were assessed applying an analysis of variance (ANOVA), followed by the calculations of post-hoc Tukey honest significant differences (TukeyHSD) (Tukey, 1984) between each pair of groups and species.

The classification process of the modern reference collection was performed using LDA that minimizes intra-group variation and maximizes between groups variation (Baylac and Frieß, 2005; Evin et al., 2013). We performed the LDA on a reduced dataset of shape variables taking into account between 9 and 12 PCs, representing 85–90% of the variance. The archaeological specimens were attributed into the known taxa using the same parameters of classification such as the number of PC axes. All analyses were performed in R (R Core Team, 2019) using "geomorph" (Adams and Otárola-Castillo, 2013), "morpho" (Schlager, 2017) and "MASS" libraries.

### 5.6. sMOR analyses of dental and postcranial remains

#### 5.6.1. Dental remains

Morphological identification of the mandibular cheek teeth was performed based on the morphological criteria identified from average tooth occlusal patterns present in the comparative reference collection. Average patterns for each equid taxa were acquired after the GPA, which superimposes the landmarks digitized on several modern individuals. A single occlusal pattern for each species group was determined by eliminating the effects of position, size and orientation of the teeth. The morphological criteria obtained from the average occlusal pattern of the teeth through GPA matched those described in previous traditional morphometric studies (Davis, 1980; Eisenmann, 1981, 1986). A detail description of the occlusal patterns used to separate different equid taxa is available in the Supplementary Text.

#### 5.6.2. Post-cranial remains

Taxonomic classification of post-cranial elements was essentially based on length, length/breadth and morphological differences previously published. Bone size can be used to separate donkeys from horses, but hybrids usually surpass donkeys in size (Johnstone, 2004; Peters, 1998). However, recently donkeys with exceptional size have also been reported in Roman times (Lepetz et al., 2021). Regarding the morphological differences among equid taxa, we observed the visual morphoscopic criteria in comparative samples on three postcranial elements: metacarpal, tibia and humerus. Metapodials represented the most reliable elements exhibiting distinctive morphological criteria, with metacarpals showing higher statistical power than metatarsals as reported previously (Eisenmann, 1986). The morphological criteria that we used in order to distinguish equid taxa were often based on previous studies of postcranial equid elements (Eisenmann and Beckouche, 1986; Johnstone, 2004; Peters, 1998). A detailed description of relevant morphological differences is available in the Supplementary Text.

## Author contributions

**Muhammad Bilal Sharif**: aDNA data generation and analysis, Visualization, Writing – original draft preparation. **Azadeh Fatemeh Mohaseb**: GMM and sMOR data generation and analysis, Writing – original draft preparation. **Michaela Isabell Zimmermann**: sMOR and GMM data generation and analysis, Writing – original draft preparation. **Simon Trixl:** sMOR analysis, Resources. **Konstantina Saliari:** Resources. **Günther Karl Kunst**: Resources, Writing- Reviewing and Editing. **Thomas Cucchi**: Conceptualization. **Sigrid Czeika**: Resources. **Marjan Mashkour**: Conceptualization, Writing- Reviewing and Editing. **Ludovic Orlando**: Conceptualization, Writing- Reviewing and Editing. **Katrin Schaefer**: Supervision, Writing- Reviewing and Editing. **Joris Peters**: Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft preparation, Reviewing and Editing. **Elmira Mohandesan**: Conceptualization, Funding acquisition, Supervision, Writing – original draft preparation, Reviewing and Editing.

## Data availability

The raw Sequencing data can be found on the European Nucleotide Archive, ENA: PRJEB52590. Additional information such as Zonkey analysis report and mapDamage graphics are available in the project website (https://mohandesandb.univie.ac.at/EQUID_DATABASE/index.php, username/password: guest) and upon request from the corresponding author [EM]. The raw coordinates of 2D and 3D GMM for all samples can be found in the Supplementary GMM coordinates file.

## Declaration of comepting interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2022.105624.

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