The mules that are not mules-metrics, morphology, archaeogenomics and mtDNA d-loop diversity in equids from Roman Switzerland

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The mules that are not mules - metrics, morphology, archaeogenomics and mtDNA d-loop diversity in equids from Roman Switzerland

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- Zonkey

ABSTRACT

Mules (Equus asinus x Equus caballus) represent first-generation hybrids between a female horse (mare) and a male donkey (jack). They are generally considered to have first appeared north of the Alps with Roman influence, a time period in which written and iconographic sources support their key role for transport and traction, both in farming and the military. The archaeozoological evidence for mules is, however, contentious as faunal assemblages are difficult to assign to either parental species or hybrids based on morphometric data alone. Here we leverage low-coverage DNA sequence data and Zonkey computational analyses to assess the occurrence of mules within Roman equid faunal assemblages in the alpine foreland. While morphological data previously assigned 17 remains to mules, successful DNA analysis of 12 remains revealed that 11 were in fact horses, one female and ten males. Eight mtDNA d-loop haplogroups were identified and genetic diversity within Roman equids corresponds to non-threatened modern local breeds. Two remains genetically identified as mules belonged to haplogroups F and I. Our results suggest that the importance of mules in the Roman archaeological record of the alpine foreland, and probably elsewhere, may have been previously over-estimated. Whether this is true for other regions of the Roman Empire needs to be evaluated. Further genomic testing for equid species and their hybrids and molecular sexing will improve our knowledge on this important issue.

1. Introduction

Classical texts, art and archaeozoological remains support a widespread socio-economic importance of equids (ie horses, donkeys and mules) in the Roman Empire (Johnstone, 2004; Chuang, 2016; Mitchell, 2018). During Roman times, a broad variety of equids showing differences in sizes and morpho-anatomy in archaeozoological assemblages co-existed (Peters, 1998; Johnstone, 2004). This is also true for the alpine foreland where domestic horses have been reported since the Bronze Age and where evidence for the presence of donkeys and mules can be found throughout the Roman period (Willms, 1990; Benecke, 1994; Schibler and Frurger, 1988; Deschler-Erb et al., 2002).

Roman horses (Equus caballus) were mainly used for riding, warfare, hunting and chariot racing. In contrast, donkeys (Equus asinus) were used as farm and pack animals, and were essential for both short-distance and long-distance transportation. Mules were also especially valued for their strength, resilience, stamina, sure-footedness on narrow mountainous paths, as well as their even temper and perseverance (Campbell Smith, 2008). Moreover, their hooves were harder than those of horses, which eliminated the need for shoeing (Jacobs and Bachmann, 2018). Mule production, however, required considerable financial investment. This is so because mules are infertile (although see Steiner and Ryde, 2013 for exceptions), and could not be sustained generation after generation. This implied the parallel maintenance of both equine parental stocks. A further limitation pertains to the number of foals that a single mare can deliver during her lifespan, which is limited to only a few individuals (Johnstone, 2004).

Overall in the Roman colonies, the relative proportion of equine remains in bone assemblages is usually below 10%, e.g. (Schibler and Frurger, 1988; Peters, 1998; Johnstone, 2004; Chuang, 2016; Wright et al., 2019). This has several reasons: equine meat was not part of the Roman middle- and upper-class diet (Peters, 1998; Deschler-Erb, 2005;
2. Material and methods

A total of thirty-three equid samples were collected from different archaeological sites from Switzerland, excavated over many years and identified by different archaeozoologists (Table S1). The selection of material was on the basis of availability and identification of species and hybrid.

2.1. Archaeological sites (Fig. 1)

2.1.1. Solothurn Vigier-Häuser

The vicus of Salodarum (today Solothurn), dated between the 1st-4th century AD, was located at one of the rare places where a bridge could be built across the river Aare. There important trade routes went through connecting Aventicum, the capital of the tribe of the Helveti, the colony Augusta Raurica and the legionary camp of Vindonissa (Flutsch et al., 2002). During the so-called “Vigier” excavations in the 1980s parts of the embankment constructions have been found which had been built for protection against flooding of the river (Backman, 1985). Faunal remains are dominated by cattle, but there is also a relatively high concentration of mostly non-fragmented equid bones, which can be interpreted as carcass disposal of worn-out pack animals (Breuer et al., 1981 unpublished and personal information).

2.1.2. Augst/Kaiseraugst/Augusta Raurica: Schmidmatt, Auf der Wacht, Theater NW, Amphitheater, Kursenbettli Mansio

The colonial town of Augusta Raurica was a large Roman city and later military camp at the river Rhine, dating to the 1st – 4th cent. AD. The city flourished until ca 200 AD with a population of over 10,000 people (Berger, 2012; Bossart et al. 2006). Rich bioarchaeological assemblages from different contexts have been excavated and analysed, e.g. from the theatre in the centre of the city (Theater-NW-Ecke) (Furger, 1988; Furger and Deschler-Erb, 1992), the amphitheatre (Gradel, 1989), the Kurzenbettli area (Schibli and Furger, 1988), the Schmidmatt area (Müller, 1985; Ginella personal communication), the well house in insula 8 (Schmid et al. 2011) and the area “Auf der Wacht” (Mráz, 2019).

2.1.3. Stein am Rhein Charregass

Stein am Rhein is located in the canton Schaffhausen, Switzerland. The site is a late Roman military camp (end 3rd – 4th cent. AD) on a hill and meant to protect the crossing of the river Rhine. Animal bones were retrieved from a ditch around the camp among them relatively high frequencies of equid bones (Flutsch et al. 2002; Ginella personal communication).

2.1.4. Eschenz

The vicus Tasgetium is located at the river Rhine and is a small town with domestic commerce/industries. It was founded in the 1st cent. AD as an important trading centre and abandoned in the 3rd cent. AD (Benguerel et al. 2011). The equid bone was found in ditch 1 Pos. 208 (Benguerel et al. 2014). Equid bones have been found rather rarely in the vicus, but they often stem from waterlogged conditions.

2.1.5. Zürich Letzigraben

The site is located in Albisrieden, a district of Zürich, Canton Zürich, Switzerland. The site was an early modern gallow and knacker yard dating to the 18th cent. AD. Nearly complete equine skeletons were recovered in foundation trenches of a former Roman villa (Deschler-Erb and Stopp, 2006).

2.1.6. Natural history Museum Bern (NHM)

One sample was obtained from a mule (1982–86) named “Negro” used by the Swiss military that was stored in the NHM Bern and originated in Visp (canton Wallis, Switzerland).

2.2. Methods

2.2.1. Morphological and metrical identification criteria of horses, donkeys and mules

The methods used to identify the Swiss equid bones morphologically and/or metricaly are quite heterogeneous (Table S1).

One group of the equid bones has been determined only morphologically (e.g. Peters, 1998; Uerpmann pers. comm.). For another part no morphological differentiation of horse and mule has been attempted or
no determination was possible due to high fragmentation. In Table S1 such cases are quoted as “equid” or “horse/equid” if the authors listed “horse” without providing a detailed explanation (Table S1).

Breuer et al. (1981) (unpubl.) were the first to use metrical determination methods working on the Solothurn-Vigier material. They expanded the log ratio diagrams of Eisenmann/Bekouche 1986 with data from the IPAS osteological reference collection (see Table S1 and Fig. S1), and subsequently included the metapodial measurements of the Solothurn-Vigier equids. They also used morphological criteria for these bones or bones belonging to the same individual. This combined method has also been used later for the determination of equid metapodials from Augusta Raurica and Zürich-Letzibad (Fig. S1).

Withers heights are calculated with the factors of Kiesewalter using the horse as the reference (Von den Driesch and Boesseneck, 1974).

2.2.2. Ancient DNA analyses implemented in Basel

Lab work with ancient samples was carried out according to strict criteria for authenticity established in the field and at IPAS Basel (Elsner et al. 2014).

We analysed 31 Roman equine samples, which were supplemented with two recent mules collected from 18th-20th cent. AD collections and used as references (Tab S1). Sample preparation and DNA extraction was undertaken according to Schibler et al. (2014) using the Qiagen DNeasy “Blood and Tissue kit” with the protocol for less than 100 mg bone powder. PCR was carried out according to Elsner et al. (2016), for primer information see Table S2. A total of 24 of these samples including all potential Roman mules, the donkey (EQ31) and the two recent mules were additionally processed at the centre of GeoGenetics, Copenhagen (Table S1 and see below).

PCR products were gel-purified with Qiagen “MinElute Gel Extraction Kit” and directly sequenced with tailed primers (Binladen et al. 2007) by Microsynth Balgach, Switzerland. Sequences were aligned with Bioedit, a consensus was built following the majority rule. Haplotypes and haplogroups were identified following the nomenclature provided by Cieslak and colleagues (Cieslak et al. 2010) and for the donkey after Kimura et al. (2011).

Diversity and population measures were calculated with Arlequin 10.0 (Excoffier and Lischer, 2010) and include hotspots within the 323bp d-loop fragment. A median joining network was deposited to GenBank under accession numbers no MN551320-551346.

2.2.3. Ancient DNA analyses implemented in Copenhagen

Laboratory handling of the samples was performed using strict ancient DNA guidelines and was carried out in the state-of-the-art ancient DNA facilities of the Centre for GeoGenetics, National History Museum, University of Copenhagen (Denmark). The emphasis for shotgun sequencing was on potential mules, few morphometrical horses were included for reasons of controls for the method.

The 24 bone samples were first mechanically cleaned on low speed, using a diamond drill. Subsequently, a small bone piece between 330 and 780 mg was cut out and pulverised using the Braun Biotech Mikro-Dismembrator S (~60 s, 3000 rpm).

DNA extractions were then carried out following the method Y as described in (Gamba et al. 2016), with slight modifications. The bone powder was first pre-digested at 37 °C for 1 h, using 4 ml of freshly prepared lysis buffer consisting of EDTA (0.45M), N-lauryl sarcosyl (0.5%) and Proteinase K (0.25 mg/ml). Afterwards the supernatant was removed and stored. The non-digested fraction of the pellet was then digested following overnight incubation at 42 °C in 4 ml lysis buffer. Both digestion steps were carried out under permanent rotation of the samples. The digestion supernatant was then collected through centrifugation (2 min, 12,000 rpm) and used for DNA extraction, following a concentration step to 250 μl using an Amicon Ultra centrifugal filter (Ultra-4, 30 kDa) and a further purification step through Qiagen MinElute columns. The DNA extract was eluted in 60 μl of Elution Buffer (EB) (10 mM Tris-Cl, pH 8.5) mixed with 0.05% Tween.

A fraction of 22.75 μl of the DNA extract was then incubated for 3 h mixed with 7 μl of USER™ Enzyme mix (NEB®) at 37 °C in order to remove uracil residues and reduce the impact of post-mortem cytosine deamination on subsequent downstream analyses.

Illumina blunt-end DNA libraries were prepared following the procedure from (Meyer and Kircher, 2010), modified by (Gamba et al. 2016). The minimal number of cycles for PCR amplification was obtained by qPCR (quantitative real-time Polymerase Chain Reaction), using the LightCycler 480 instrument (Roche) and a 1:20 dilution of each library. The user-treated libraries were then amplified for 5 to 12 cycles, following the protocol used in (Gamba et al. 2016), with a final PCR reaction volume of 25 μl and using 3 μl of non-purified DNA. All PCR products were purified using the Qiagen MinElute kit and an elution volume of 25 μl. Subsequent quantification of 1:10 dilutions of the amplified libraries was carried out on the Tapestation 2200 instrument (Agilent). At last the purified DNA libraries were pooled equimolarly and sequenced at the Danish National High-Throughput DNA Sequencing Centre on the HiSeq2500 (Illumina).

Computation was done with the open-source Zonkey pipeline implemented in PALEOMIX (Schubert et al. 2017).

Note: In the following text, tables and figures we will use the term “horse” instead of horse/equid for archaeozoological material for the sake of convenience and because both mules and horses cannot be distinguished at the mitochondrial level.

3. Results

3.1. Morphological and metrical analysis

A taxonomic status was assigned to 31 Roman equid samples, applying archaeozoological criteria based on morphology, measurements and ratio diagrams (Von den Driesch, 1976; Eisenmann and Beckouche, 1986; Peters, 1998) (Table S1, Fig. S1). A total of 17 could be identified as mules (EQ1-EQ17, mule group), three as domestic horses (EQ18-EQ20; Equus caballus, horse group), ten as horse/equid (EQ21-EQ30, horse group) and one single specimen as a donkey (Equus asinus). In addition, one 18th cent. AD nearly complete skeleton was identified as belonging to a mule (Deschler-Erb and Stopp, 2006) (Table S1, Fig. S1). Morphological sex identification was not attempted in the absence of strong sexual dimorphism. Besides, the presence of canine teeth does not unequivocally single out male individuals. Mules are expected to be larger than their parent species (Mitchell, 2018), however the heights at the withers of 13 Roman specimens identified as mules were calculated to range between 132 cm and 148 cm, which represents rather small animals compared to other equids from Roman sites in the area (Deschler-Erb 1998, 2009) and within the Roman Empire, e.g. (Johnstone, 2004).

3.2. mtDNA d-loop and archaeogenomics of equids

3.2.1. Roman equids: results of low-depth shotgun sequencing and mtDNA d-loop haplotype identification

Low-depth shotgun sequencing of the equine genome revealed that 11 out of 12 animals morphologically identified as mules were in fact mis-identified and represent horses instead (Table S1, Fig. 2). All except one were males. Three further individuals were most-likely assigned to mules, although the assignment is only tentative due to the limited amount of sequence data generated (% endogenous DNA content: 0.024–0.04) and failed mtDNA amplification attempts. For two individuals the genetic analysis failed. From 13 individuals morphologically identified as horses, four were tested and three were confirmed to be a horse by shotgun DNA sequences and Zonkey (Table S1, Table 1,
Fig. 2). One (EQ28, JG 156) was genetically revealed to be a female mule. In total, low-depth shotgun genomic sequencing identified only two mules in the Roman osseous assemblage investigated (Fig. 2).

Amongst the 11 horses that were morphologically mis-identified as mules, mtDNA haplotypes belong to four main equine mtDNA haplogroups: X2, D/D3, B1 and X3c1. The single animal morphologically and genetically identified as a mule (NB14) was a male and carried the F mtDNA haplogroup. Furthermore, the female mule (EQ28, JG 156) belonged to the mtDNA I haplogroup (Table 1).

Altogether the genetically identified horses and morphometrically identified horses belonged to eight haplogroups (A, B, B1, I, F, X2, D/D3 and X3c1) which encompass a large range of equine mtDNA variation (Table 1, Fig. 3). In total in 6 out of 30 (17 Roman mule group and 13 Roman horse group), mtDNA d-loop typing failed.

3.2.1. The Roman donkey. The single individual (EQ31, JG84) that was assigned morphologically as a donkey was genetically confirmed to be a donkey. This animal was excavated at Solothurn Vigier, and represented a donkey jack that carried one haplotype belonging to the Nubian/African mtDNA lineage haplotype (Table 1, Table S1, Fig. S2) (Kimura et al. 2011).

3.2.2. Early modern and modern equids.

One modern Swiss military mule (EQ33, NB8) was genetically confirmed to be a mule, and carried mtDNA haplogroup D/D3. Surprisingly, the 18th century mule JG77, best documented morphometrically as a mule based on several measurements of the same individual (Deschler-Erb and Stopp, 2006) turned out to be a male horse, belonging to haplogroup K2a (Table S1, Fig S1, Table 1, Fig. 2).

3.3. mtDNA d-loop diversity and network analysis

We next investigated whether the maternal lineages within horses of the mule group displayed different patterns of genetic diversity than
Table 1
Diagnostic SNPs of Roman time and Early modern equids in comparison with X79547 (Xu and Anarson, 1994) between position 15492-15659 (NB samples) 15492-15669 (JG samples) and 15696-15759 (all samples). Haplotypes are according to (Cieslak et al. 2010). Positions corresponding to mutational hotspots 15585, 15597, 15604, 15650 are excluded. See Table S1 for details of identification.

<table>
<thead>
<tr>
<th>Sample</th>
<th>15' ... mtDNA d-loop haplotype</th>
<th>Roman time equids</th>
<th>archaeozoology species after Zonkey</th>
<th>species after archaeozoology species after Zonkey</th>
</tr>
</thead>
<tbody>
<tr>
<td>EQ2 NB02</td>
<td>mule</td>
<td>failed, unknown</td>
<td>494C; 495G; 496G; 534T; 602T; 603G; 649G; 720A 494C; 495G; 496G; 534T; 602T; 617C; 659C; 720A</td>
<td>X2</td>
</tr>
<tr>
<td>EQ3 NB03</td>
<td>mule</td>
<td>horse</td>
<td>494C; 495G; 602T; 617C; 659C; 720A 494C; 495G; 602T; 617C; 659C; 720A</td>
<td>B1 + 494C</td>
</tr>
<tr>
<td>EQ5 NB05</td>
<td>mule</td>
<td>horse</td>
<td>495C; 720A 495C; 720A</td>
<td>D/D3</td>
</tr>
<tr>
<td>EQ10 NB10</td>
<td>mule</td>
<td>horse</td>
<td>495C; 720A 495C; 720A</td>
<td>D/D3</td>
</tr>
<tr>
<td>EQ11 NB11</td>
<td>mule</td>
<td>horse</td>
<td>495C; 720A 495C; 720A</td>
<td>D/D3</td>
</tr>
<tr>
<td>EQ12 NB12</td>
<td>mule</td>
<td>horse</td>
<td>494C; 495G; 496G; 534T; 602T; 603G; 649G; 720A 494C; 495G; 534T; 602T; 603G; 649G; 720A</td>
<td>X2</td>
</tr>
<tr>
<td>EQ14 NB14</td>
<td>mule</td>
<td>mule</td>
<td>495C; 584T; 601C; 602T; 603C; 649G; 720A 495C; 584T; 601C; 602T; 603C; 649G; 720A</td>
<td>F + 584T</td>
</tr>
<tr>
<td>EQ15 NB15</td>
<td>mule</td>
<td>horse</td>
<td>494C; 495C; 496G; 534T; 602T; 603G; 649G; 720A 494C; 495C; 496G; 534T; 602T; 603G; 649G; 720A</td>
<td>X2</td>
</tr>
<tr>
<td>EQ16 NB16</td>
<td>mule</td>
<td>horse</td>
<td>494C; 495C; 496G; 534T; 602T; 603G; 649G; 720A 494C; 495C; 496G; 534T; 602T; 603G; 649G; 720A</td>
<td>X2</td>
</tr>
<tr>
<td>EQ7 JG78</td>
<td>mule</td>
<td>not determined</td>
<td>720A 495C; 542T; 602T; 635T; 703C 720A 495C; 542T; 602T; 635T; 703C; 720A</td>
<td>X3c1</td>
</tr>
<tr>
<td>EQ8 JG79</td>
<td>mule</td>
<td>not determined</td>
<td>720A 495C; 666A; 720A 495C; 666A; 720A</td>
<td>D/D3</td>
</tr>
<tr>
<td>EQ24 JG56</td>
<td>horse</td>
<td>not determined</td>
<td>720A 495C; 542T; 602T; 635T; 703C 720A 495C; 542T; 602T; 635T; 703C; 720A</td>
<td>X3c1</td>
</tr>
<tr>
<td>EQ18 JG80</td>
<td>horse</td>
<td>not determined</td>
<td>495C; 602T; 720A 495C; 602T; 720A</td>
<td>A</td>
</tr>
<tr>
<td>EQ19 JG81</td>
<td>horse</td>
<td>not determined</td>
<td>495C; 602T; 720A 495C; 602T; 720A</td>
<td>B1</td>
</tr>
<tr>
<td>EQ25 JG94</td>
<td>horse</td>
<td>horse</td>
<td>495C; 602T; 720A 495C; 602T; 720A</td>
<td>B</td>
</tr>
<tr>
<td>EQ26 JG98</td>
<td>horse</td>
<td>horse</td>
<td>495C; 602T; 720A 495C; 602T; 720A</td>
<td>D/D3</td>
</tr>
<tr>
<td>EQ27 JG99</td>
<td>horse</td>
<td>not determined</td>
<td>494C; 495C; 496G; 534T; 602T; 603C; 649G; 720A 494C; 495C; 496G; 534T; 602T; 603C; 649G; 720A</td>
<td>X2</td>
</tr>
<tr>
<td>EQ28 JG156</td>
<td>horse</td>
<td>mule</td>
<td>495C; 538G; 602T; 703C; 709T; 720A 495C; 538G; 602T; 703C; 709T; 720A</td>
<td>I + 703C</td>
</tr>
<tr>
<td>EQ29 JG151</td>
<td>horse</td>
<td>not determined</td>
<td>495C; 602T; 720A 495C; 602T; 720A</td>
<td>B1</td>
</tr>
<tr>
<td>EQ30 JG154</td>
<td>horse</td>
<td>not determined</td>
<td>495C; 602T; 720A 495C; 602T; 720A</td>
<td>F</td>
</tr>
<tr>
<td>EQ20 JG157</td>
<td>horse</td>
<td>not determined</td>
<td>495C; 602T; 720A 495C; 602T; 720A</td>
<td>F</td>
</tr>
<tr>
<td>EQ21 JG159</td>
<td>horse</td>
<td>not determined</td>
<td>495C; 538G; 602T; 703C; 709T; 720A 495C; 538G; 602T; 703C; 709T; 720A</td>
<td>I</td>
</tr>
<tr>
<td>EQ22 JG160</td>
<td>horse</td>
<td>horse</td>
<td>494C; 495C; 496G; 534T; 602T; 603C; 649G; 720A 494C; 495C; 496G; 534T; 602T; 603C; 649G; 720A</td>
<td>X2</td>
</tr>
<tr>
<td>EQ23 JG163</td>
<td>horse</td>
<td>not determined</td>
<td>495C; 538G; 602T; 703C; 709T; 720A 495C; 538G; 602T; 703C; 709T; 720A</td>
<td>I</td>
</tr>
<tr>
<td>EQ33 NB08</td>
<td>mule</td>
<td>mule</td>
<td>495C; 602T; 720A 495C; 602T; 720A</td>
<td>D/D3</td>
</tr>
<tr>
<td>EQ32 JG77</td>
<td>mule</td>
<td>horse</td>
<td>495C; 595G; 602T; 703C; 720A 495C; 595G; 602T; 703C; 720A</td>
<td>K2b</td>
</tr>
<tr>
<td>Equation donkey</td>
<td>JG84 donkey</td>
<td>donkey</td>
<td>495C; 602T; 720A 495C; 602T; 720A</td>
<td>740G</td>
</tr>
</tbody>
</table>

(continued on next page)
those of the horse group (Table S1) which might explain the misidentifications. Both groups showed similar nucleotide diversity (π), however, the haplotype diversity of the horses that were morphologically mis-classified as mules was reduced and even more reduced if only males are considered (Table 2). The Roman horses investigated here showed similar nucleotide diversity compared to Celtic horses from the same region (Elsner et al. 2016), but a slightly higher haplotype diversity (Table 2). Most horses belong to the three major haplogroups F, D/D3 and X2. Haplotypes of haplogroups F, B1 and D/D3 are shared between Iron Age and Roman period (Fig. 3). No identical maternal lineages of the two mules was found in the dataset.

4. Discussion

The taxonomical assignment of equine bone assemblages at the species level can prove especially hard in the presence of fragmentary pieces and overlapping morphological ranges for particular skeletal elements (Deschler-Erb, 2009; Schubert et al. 2017). Another factor that makes morphological identification even more complex is the presence of hybrids, such as mules and hinnies. Hybrids are known both in mammals, but also in birds and reptiles, for their highly variable morphology, which is not necessarily intermediate relative to both parental species (Bocheński and Tomek, 2000; Bochaton et al. 2015). Therefore, morphological criteria developed within one assemblage cannot be consequently extended to another and morphological predictions should ideally be systematically confirmed with molecular approaches such as the one implemented here before definitive conclusions on the relative use of equine animals in a particular archaeological context can be drawn.

Previous morphological and morpho-metrical work already proposed that mules were less abundant than horses in Roman sites, e.g. in Switzerland and Germany (Deschler-Erb, 2009). This observation was confirmed in our study. The extent of morphological mis-identification was, however, unexpected. It is noteworthy that it concerned male individuals in all but one case. The height at the withers of those mis-classified animals was inconspicuous and fell within the range recorded for Roman horses around Europe (Groot, 2009), but was also consistent with the estimates obtained for a mule from Kalkriese (130 cm–140 cm) (Uerpmann and Uerpmann, 2007) and the early modern mule from the Swiss army analysed in this study (NB08). Today, such animals would be regarded as ponies.

The group of horses that were mis-classified as mules was found to harbour lower haplotype diversity compared to the remaining horses but similar nucleotide diversity. They carried the main mtDNA haplogroups F, D/D3 and X2, all of which are common today and since prehistory (Cieslak et al. 2010). Altogether, mtDNA d-loop diversity of Roman period horses was similar to regional Iron Age horses (Elsner et al. 2016), and shared most haplogroups with them. This might imply, that Roman influence is not reflected in mtDNA d-loop diversity. Several haplotypes of haplogroups F, B1 and D/D3 are shared between Iron Age and Roman period (Fig. 3). No identical maternal lineages of the two mules was found in the dataset.

Table 2

<table>
<thead>
<tr>
<th>Species identified as</th>
<th>Sample</th>
<th>15'…</th>
<th>mtDNA d-loop haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roman period horses</td>
<td>626G;</td>
<td>Africanus</td>
<td></td>
</tr>
<tr>
<td>horses, CH, EQ1-EQ30</td>
<td>63ST;</td>
<td>lineage</td>
<td></td>
</tr>
<tr>
<td>667G;</td>
<td>donkey</td>
<td></td>
<td></td>
</tr>
<tr>
<td>719C;</td>
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Fig. 3. Median joining network of the 232 bp mtDNA d-loop of equids (this paper, NB and JG) in comparison with Iron Age horses from Switzerland (MO, BGF, AV, Table S3). The network is based on six haplotypes. N = number of individuals, nHt = number of haplotypes, Htdiv = haplotype diversity, MNPD = mean number of pairwise distances. Note that the two mules identified by Zonkey are not included.
new haplotypes appeared with the Roman period, which might indicate the import of new maternal lineages. Based on the still limited dataset, further research is, however, needed to clarify the issue. Compared to other modern horse breeds the mtDNA d-loop diversity of Roman horses from Switzerland are similar to breeds which are thriving and are not endangered (Elser et al. 2016). Regrettably only scarce data from other regional Roman contexts are available so far. For example, only three individuals were sequenced from Roman Dangstetten (Schubert et al. 2017) and other studies on horse ancient DNA cover only few individuals within a large geographic area and include no Roman time individuals, e.g. (Cieslak et al. 2010; Lira et al. 2010). Here, we report the presence of a genetically confirmed male donkey at Solothurn-Vigier dated to the 1st cent AD. Interestingly, this animal carried a mtDNA haplogroup previously identified amongst the Chalcolithic bone assemblages of Portugal (Cardoso et al. 2013). The earliest mule genetically identified in the archaeological record was found in La Tene sites from France and radiocarbon dated to ~2300 years ago (Pages et al. 2019). Based on morphometrics mule breeding was suggested in Northern Iberia in the 3rd – 2nd cent. BC (Albiziri et al. 2017). Determining whether mule breeding also developed at that time north of the Alps requires further DNA analyses. For now, our work demonstrates that mule breeding was at least potentially ongoing during the 1st cent. AD in the region, although identical maternal lineages of the two mules F+584T and I+703C were so far not detected in the horses from Roman time. Members of haplogroup F and I are today most frequent in the Balkans (Cieslak et al. 2018).

Because the majority of animals that were classified morphometrically as mules were horse males, it could be hypothesized that these animals in fact represent castrates. Castration was well known in Roman time and described by Varro, Columella and Pliny (Peters, 1998). Castration is aimed at limiting the strong herd behaviour and sexual aggression present in horse stallions so as to facilitate husbandry. The removal of testicles early in life, as done today, shows a range of bio-physiological consequences, including the reduction of testosterone production, leading to skeletal changes such as delayed epiphyseal fusion, bone proportion shifts (prolonged growth) and metaphysal thickening, especially on metapodials, and larger body height and more body fat. This is well documented for cattle and goats (Shahin et al. 1993; Davis, 2000; Telldahl et al. 2011). Similar effects are also suspected for the horse (Martin-Rosset and Juliard, 2005; Omar et al. 2018; Nistelberger et al. 2019) but remain undocumented. However, if, as reported in some textual sources, Romans performed castration later in age (Johnstone, 2004), the effect on body shape might not be very pronounced and would fit the average wither heights of the male horses of this study. Further work is, however, necessary before castration can be definitively ruled out.

5. Conclusion

We show a discrepancy between morphological/metric analyses and shotgun genome sequencing of equid species and hybrids. In particular, a large number of mules were genetically identified as male horses, although one congruent identification exists and one mule was mis-identified as horse. The horses misidentified as mules do not differ in height or mtDNA genetic diversity to those identified as horses. Roman horses from Switzerland were found to be as diverse as Celtic horses from the area and a few continuous haplotypes were detected. The only two mules, however, carry maternal lineages not present in the dataset. Although this study focussed on a local scale using Germania superior as an example, it has impact on other regions, as the identification of equid species and hybrids is a general methodological problem. The question as to why morphology of mules appears to be confounded particularly with male horses has to be answered in the future. Species identification is also an issue in relation to other wild and domestic fauna, e.g. aurochs and domestic cattle or the sheep-goat problem. Employing shotgun sequence data does also inform about e.g. the sex of an individual giving clues to the value of male and female animals in past societies.

This work has demonstrated the unreliability of the archaeological identification criteria used to identify mules in the archaeological record, and indicates that it is generally safer to make identifications at the genus level unless they can be confirmed using genetic methods, or until new, more reliable morphological identification methods can be developed. Genetic approaches, alongside a number of other biomolecular techniques, such as ZooMS, are now revolutionising our ability to deal with issues around species identification, and ultimately this means that we are able to better understand animal economies in the past.

Declaration of competing interest

None.

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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.2020.105253.

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