Journal of Archaeological Science: Reports 6 (2016) 386–396

Contents lists available at ScienceDirect

Journal of Archaeological Science: Reports

journal homepage: www.elsevier.com/locate/jasrep

Mitochondrial d-loop variation, coat colour and sex identification of Late Iron Age horses in Switzerland

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ARTICLE INFO

Article history:
Received 26 November 2015
Accepted 7 March 2016
Available online 14 March 2016

Keywords:
Genetic diversity
Ancient DNA
Iron Age
Horse
Coat colour
Withers height
Greying
Sex identification

ABSTRACT

In the Celtic world, horses enjoyed a prominent position as status symbols and objects of veneration, yet little is known about these Celtic horses except that they were rather small. The Late Iron Age was a time defined by increasing inter-cultural contact between Celtic peoples and the Romans. This is, amongst other features, observable in the phenotypes of domestic livestock such as horses. Amongst the usually small animals, larger ones are rarely but regularly encountered in the archaeological record. We have investigated mitochondrial (mt) DNA d-loop diversity, sex and coat colour using bones from 34 horses of different size from three Swiss sites (Mormont, Basel-Gasfabrik, Aventicum) most of them dating from 150 to 50 BCE. The aim was to characterise the diversity of matrilineages and coat colourations of Iron Age horses, and to identify molecular sex. We detected eleven mt haplotypes clustering into six haplogroups (B, D, F, I, X2, X3) in the ancient dataset (n = 19). Large individuals were all male, but smaller stallions were also identified; molecular sexing confirmed and augmented to morphological results. The horses were bay, chestnut and black in colour, and spottings or dilutions were absent in all animals. With a simplified primer system to detect premature greying, white coats can be excluded as well. The limited colour range proposes selection for monochrome animals. Additionally, ancient matrilineages were compared to modern horses from regions appertaining to the Late Roman Republic and to European pony breeds. Based on Principal Component Analysis (haplotype frequencies) and F_{ST}-values (genetic distances) the mtDNA variation of the Iron Age horses investigated here has survived in modern European breeds, particularly in northern European ponies.

1. Introduction

Several Late Iron Age sites have been identified in Switzerland, including the eponymous sacrificial site in La Tène, canton Neuchâtel. In recent years comprehensive interdisciplinary investigations were carried out at Basel-Gasfabrik (Pichler et al., in preparation) and on the hill of Mormont (Brunetti et al., 2014) to gain detailed insight into the socio-economy of Celtic people in the region. In the Late Iron Age, contacts between Celtic groups and Romans intensified. Mainly the Celtic nobility were susceptible for Roman influence and long distance trade increased in the alpine foreland (Brem and Haldimann, 1999) to import prestige items like wine and tableware, and, according to Caesar, also horses (bell. gall. 4,2,2: Möller, 2013). Several written sources describe ancient horse and mule trade across Europe, Asia and Africa (see e.g. Peters, 1998: 140f), yet the scientific evidence for this is scarce (Paulus and Uerpmann, 2007; Nuviala et al., 2014). Horses were venerated status symbols in ancient times (Green, 1992); they were significant for transportation and warfare; however, horse meat was also eaten on a regular basis by the Celts. Horses played an important part in Celtic symbolism as well, apparent in Epona, patroness of horses and riders, and in the ritual life as indicated for example from perforated horse skulls from La Tène (Méniel, 2007) or carcass depositions in Gournay-sur-Aronde, France (Rapin et al., 1980). However, most Iron Age sites in Switzerland do not contain more than 5% horse remains (Schibler et al., 1999: 121). Celtic horses are described as small (110–130 cm withers height), whereas the Romans also bred larger animals (>140 cm) (Müller-Lhotska, 1984: 114; Peters, 1998: 149f; Arbogast et al., 2002: 44f). Therefore, when encountering small and large horses in Celtic contexts, archaeozoologists often consider the small ones to be local, while larger ones are regarded as imported, endowed or looted. Some authors argue that the reason for the small size of the Celtic horse was poor pasture and low breeding interest (Boessneck et al., 1971: 106), but this interpretation contradicts the Celts' reputation as stock breeders and mounted warriors (Hyland, 1990: 173; Green, 1992: 66; Sidnell, 2006: 162). As small horses are more versatile and quick on the battlefield (Junkelmann, 1990: 44), it can be assumed that the Celts intentionally bred small horses. However, by the Late Iron Age, small and large horses were found to be coexisting in Celtic sites for example in southern Germany (Manching) or Luxembourg (Titelberg)

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http://dx.doi.org/10.1016/j.jasrep.2016.03.007

(Boessneck et al., 1971; Méniel, 2014: 18). Little is known about the genetic diversity of Celtic and Roman horses (Di Bernardo et al., 2004; Bower et al., 2013), yet it is speculated that the Romans introduced new female lineages to conquered regions (Bower et al., 2013).

In ancient DNA (aDNA) studies, the analysis of the non-recombining maternally inherited mitochondrial (mt) DNA has been widely applied due to its high copy number in the cell and the high variability within the non-coding displacement-loop (d-loop). Studies of modern horse mtDNA diversity have revealed an intricate phylogenetic structure. Based on 616 base pairs six major haplogroups were defined (Vila et al., 2001; Jansen et al., 2002). The inclusion of additional aDNA data (Cieslak et al., 2010) and whole mitochondrial genomes (Lippold et al., 2011b; Achilli et al., 2012) led to revisions of the initial nomenclature, resulting in now 18 or 19 haplogroups. Apparently, geographic structuring is low today, and was so in wild horse populations, but there is also evidence that some matrilineages were regionally confined (Cieslak et al., 2010; Lira et al., 2010). Studies featuring ancient horse mtDNA suggest multiple domestications and an increasing diversity of female lineages from the Bronze Age onwards (Vila et al., 2001; Cieslak et al., 2010).

Animals with unique colour variants may have been specially valued. Individual phenotypic information can be retrieved from nuclear DNA, e.g. from single nucleotide polymorphisms (SNPs). An increasing number of genetic loci associated with coat colour has been detected so far (for reviews see Rieder, 2009; Cieslak et al., 2011) including the pleiotropic effect some of them have on behaviour and health (Reissmann, 2009: 18f; Trut et al., 2009; Bellone, 2010).

Morphological sex identification in archaeological horse remains is feasible when determinant skeletal elements are preserved; these include the canines, which dominantly prevail in males, and the pelvis (Montane et al., 1913). Molecular sex identification is possible using the amelogenin gene (Iwase et al., 2003) and the zinc-finger gene on the X and Y chromosomes (Senese et al., 1999; Han et al., 2010).

Here we investigate horse remains, mostly with known withers heights, from three key Late Iron Age sites in Switzerland where Roman cultural influence is noticeable in the archaeological record. We explore mitochondrial d-loop variation, molecular sex and coat colour markers of 32 Late La Tène and two Roman time horses. The diversity of the ancient female lineages is compared to modern indigenous horse and pony breeds. This is the first study focusing on variation in horses from a defined region inhabited by Celtic peoples, zooming in on a narrow time frame.

2. Material and methods

2.1. Sites and material

We have selected 32 horse remains from Late Iron Age contexts (La Tène D, 150 BCE–30 CE), the sites of Mormont, Basel-Gasfabrik and pre-Roman Aventicum in Switzerland (Fig. 1). Two samples dating to the late 1st to early 2nd century AD from Aventicum have been added to the analysis. Sample details are given in Table 1. All sites comprised more horse remains than investigated here.

2.1.1. Mormont

The site on the hill of Mormont near Eclépens and La Sarraz, canton Vaud, was first discovered in 2006 during gravel works and addressed as a Helvetic sanctuary (Dietrich et al., 2007). After examining the faunal remains, a second scenario was proposed: the site might have been a temporal camp of refugees or migrants (Méniel, 2014: 195f). Since the investigations by other archaeological disciplines are not finished yet, both interpretations have to be regarded as preliminary. According to typological dating the main occupation time of the site was La Tène D1b (130/120–90/80 BCE), which is supported by absolute dendrochronological dates between 107 and 102 BCE (Kaenel, 2014). During this time c. 300 structures were constructed including 260 pits, filled with various items ranging from mill stones, ceramic and metal pots, and decorative objects to an abundance of faunal remains and even several human bones. Horses ($n = 40$) are the third most common animal based on minimum number of individuals (MNI), after cattle ($n = 174$) and pigs ($n = 59$) (Méniel, 2014: 12). Given the short occupation time, they might stem from contemporaneous populations. The dead animals had been treated in different ways, some were deposited as articulated (partial) skeletons or cadavers, other bones show cut marks

Fig. 1. Location of investigated sites in Switzerland. 1 – Mormont, canton Vaud; 2 – Avenches/Aventicum, canton Vaud; 3 – Basel-Gasfabrik, canton Basel-City.

Table 1
Details of archaeologic sites and samples, and summary of results. Given are name, location and date of sites, and laboratory and archaeological code, morphological sex and age identification if determinant elements (pelvis, teeth) are present, skeletal element for withers height estimation and genetic analyses, as well as mitochondrial haplotype, GenBank accession code of d-loop sequences; molecular sex and coat colour. The measurement for greatest length (GL) and smallest breadth of diaphysis (SD) are given in mm, withers height (WH) was estimated after May (1985) and is given in cm. For some of the samples from Mormont, radius GL and SD from the same individual are given in parenthesis (Méniel, 2014: 264). Details on the mitochondrial haplotypes are given in Table 5, on molecular sexing in Table 6 and nuclear loci for the coat colour analysis in Table 7. Nomenclature of mitochondrial haplotypes follows Cieslak et al. (2010).

Site, location (altitude, latitude, longitude), date	Samples										
	Lab code	Arch code	Morph. sex, age	Skeletal element	GL	SD	WH	Mitochondrial haplotype	GenBank ID	Molecular sex	Coat colour
Mormont, 605 m, 46.66/6.54, Late La Tène, c. 100 BCE	MO1	Id 4 fosse 293	Female, 18 y	Metacarpus sinister	191 (285)	27 (31)	116.5 (117)	I	KC893855	–	–
	MO2	Id 35 fosse 288	–	Metacarpus sinister	181	27	110.5	D3	KC893856	Female	–
	MO3	Id 3 fosse 42	Female, 11.5 y	Metacarpus sinister	191 (281)	25.5 (29.3)	116.5 (115.5)	–	–	–	–
	MO4	Id 30 fosse 143	–	Metacarpus sinister	199	29	121.5	D2	KC893857	–	Chestnut
	MO5	Id 21 fosse 94	Female, 15 y	Metacarpus sinister	209	30	127.5	X2b	KC893858	Female	Black/not grey
	MO6	Id 44 fosse 196	–	Metacarpus sinister	202	30.5	123	B1	KC893859	Male	Black
	MO7	Id 2 fosse 131	Female, 10 y	Metacarpus sinister	189 (281)	27 (30.5)	115 (115.5)	–	–	–	–
	MO8	Id 27 fosse 146	–	Metacarpus sinister	205	28	125	–	–	–	–
	MO9	Id 42 fosse 275	–	Metacarpus sinister	194	29	118	X3c1 + 15,615G	KC893860	Male	Black/not grey
	MO10	Id 01 fosse 45	Male, 10 y	Metacarpus dexter	241 (365)	36 (41)	147 (150)	A/B/C/D/F?	KC893861	–	–
	MO11	Id 10 fosse 146		Metacarpus sinister	Incomplete			–	–	–	–
	MO12	Id 32 fosse 146	–	Metacarpus sinister	190	28	116	F	KC893862	–	–
	MO13	Id 53 fosse 210	Male, 5.5 y	Metacarpus sinister	191 (290)	29.7 (32)	116.5 (119)	–	–	–	–
	MO14	Id 58 fosse 542	–	Metacarpus sinister	Incomplete			K?	KC893863	–	–
	MO15	Id 54 fosse 210	Male, 5 y	Metacarpus sinister	208	29.7	127	X2b	KC893864	–	–
	MO16	Id 16 fosse 205	Male, 4 y	Metacarpus sinister	234 (340)	35.5 (39.2)	143 (140)	F	KC893865	Male	Bay/not grey
	MO17	Id 57 fosse 542	–	Metacarpus sinister	194	30	118	X2b	KC893866	–	Chestnut/not grey
	MO18	Id 55 fosse 169	Male, 1.5 y	Metacarpus sinister	Incomplete (subadult)			X2	KC893867	–	–
Basel-Gasfabrik, 255 m, 47.57/7.58, Late La Tène, 150–80 BCE	BGF1	Fk22519 pit44 excavation 1990/42	–	Metacarpus	201.5	30.4	123	F	KC893850	–	Chestnut
	BGF2	Fk22576 pit284 excavation 1990/32	–	Metacarpus	222.8	35.5	136	X2b	KC893851	–	–
	BGF3	Fk″2″ pit114 excavation 1941/4	–	Metacarpus	196.9	31.4	120	X3c1 + 631G	KC893852	–	–
	BGF4	Fk″2″ pit114 excavation 1941/4	–	Metacarpus	178.1	24.9	109	–	–	–	Chestnut
	BGF5	Fk 18796 pit256 excavation 1989/5	–	Radius/Ulna	276	33	113.5	F	KC893854	–	–
	BGF6	Fk(18943)18714 pit255 excavation 1989/5	–	Upper (pre)molar		–		–	–	–	–
Avenches, 630 m, 46.87/7.05, *Late La Tène, c. 50 BCE, **Roman, late 1st to early 2nd century CE	AV1**	K10656, Sk4	–	Radius	330	35	135.5	–	–	–	–
	AV2*	K10574	–	Radius/ulna	315	36	129.5	X3c1 + 15,615G	KC893849	–	–
	AV3**	K10656, Sk3	–	Radius	358	39.7	147	X2	KC893848	–	–

* refers to AV2 and Late La Tène. ** refers to AV1, AV3 and Roman.

and traces of burning. We have examined 18 randomly chosen equid metacarpi in this study.

2.1.2. Basel-Gasfabrik

In Basel, a settlement of the Rauricii was discovered in 1911 at the left border of the river Rhine (Hecht and Niederhäuser, 2011: 6), today within the area of chemical industry (Gasfabrik, GF). It was excavated in several campaigns during the following 100 years. The unfortified proto-urban site was inhabited between 150 and 80 BCE (La Tène D1) as indicated for example by fibula types and coins. It comprised houses, gardens and pens as well as craft zones, and two cemeteries. The majority of archaeological and faunal remains stem from the c. 585 pits which had been filled with "waste" after they had become unusable as e.g. storage cellars (Hecht and Niederhäuser, 2011: 47). Five horse limb bones and one tooth were chosen for the analysis based on calculability of the horses' withers height (except for the tooth).

2.1.3. Aventicum

Avenches, canton Vaud, was once the capital of the Helvetii known as Aventicum (Castella and Meylan, 2008). Celtic temples had been built in the first century BC, and under the reign of the Roman Emperor Augustus, who forced the urbanisation of the Celtic provinces, Roman town structures were added in the first decade CE which persisted about three hundred years (Blanc and Frei-Stolba, 2001). Aventicum became a Roman colony in 71 CE and was incorporated into *Germania Superior* when the province was formed in 85 CE (Blanc et al., 2001).The three radii studied here stem from sacrificial pits nearby the temples *au Lavoëx* and were chosen due to their large size (Deschler-Erb, 2015).

2.2. Modern horse populations

The matrilineages of the ancient horses were compared to modern horse mtDNA variability. The comparison of modern animal sequences to ancient genetic data has to be treated with caution. In ancient times, there were no breeds in today's sense (Johnstone, 2004: 88f) yet different morphotypes were described, for example by Vegetius (mulom. 3,6,2: Lommatzsch, 1903). We selected breeds that come from regions belonging to the Late Roman Republic in c. 100 BCE (Crook et al., 1994: add. mat. 1), contemporaneous to the occupation of both Mormont and Basel-GF. They were grouped into: Spain (*Hispania Citerior* and *Ulterior*); Italy (*Italia*, *Sardinia*, *Gallia Cisalpina*), and Croatia (*Illyricum*). In addition, breeds from Switzerland, southern Germany and eastern France represent the home region of the Celtic horses investigated here. Breeds were chosen due to their "indigenous" status according to the Domestic Animal Diversity Information System (DAD-IS; dad.fao.org), to availability on GenBank (ncbi.nlm.nih.gov) in April 2015 and when the authors sampled material from maternally unrelated individuals. As Celtic horses are ponies by today's standards (withers heights <148 cm) (Fédération Equestre Internationale, 2015: 60) we compared them also to European pony breeds from Spain, Italy, the Carpathians, European Russia, and Northern Europe (including the British Isles). In total, we assembled sequences from 35 museum and 726 modern specimens representing 30 breeds (Table S1).

2.3. Metrics

Measurements of greatest length (GL) and smallest breadth of diaphysis (SD) of metacarpi and/or radii from Mormont were taken from Méniel (2014: 263f); and from Aventicum and Basel-GF measured according to von den Driesch (1976). Withers heights were calculated from GL of all bones according to May (1985); note that this results in an estimation disregarding sexual dimorphism or robustness.

$$\text{Metacarpus}: \quad \text{GL}\ (\text{mm}) * 0.6102 = \text{WH}\ (\text{cm})$$

$$\text{Radius}: \quad \text{GL}\ (\text{mm}) * 0.4111 = \text{WH}\ (\text{cm})$$

2.4. Sample preparation, DNA extraction and PCR analysis

Sample preparation and DNA extraction were performed as previously described in Elsner et al. (2014) following the User Developed Protocol: „Purification of total DNA from compact animal bone using the DNeasy® Blood & Tissue Kit "(Qiagen, Basel, Switzerland) in dedicated aDNA facilities physically separated from post-PCR laboratories. Standard precautions and measures in aDNA processing were adhered to (Shapiro and Hofreiter, 2012).

PCR for mtDNA and the nuclear (nc) loci (grey, *AMELX/Y*, *ZFX/Y*) was set up in 25 μl volumes containing 1.5 U AmpliTaq Gold, 1× GeneAmp 10× PCR Gold Buffer (150 mM Tris-HCl, 500 mM KCl, pH 8.0) and 2 mM $MgCl_2$ (all Applied Biosystems, Hombrechtikon, Switzerland); 0.4 mM dNTP Mix (Promega, Dübendorf, Switzerland); 0.2 μM of each primer; 20 μg/μl BSA (bovine serum albumin, Roche, Basel, Switzerland), and up to 10 μl template DNA on a Mastercycler ProS (Eppendorf, Allschwil, Switzerland). The cycling conditions were: 12 min initial denaturation, followed by either 50 (for mtDNA) or 70 (for ncDNA) cycles of denaturation at 95 °C for 40 s, annealing at 52 °C to 58 °C (see Tables 2–4) for 30 s, and extension at 72 °C for 30 s, with a final extension of 60 s at 72 °C. Non-template controls were performed alongside all amplifications. Amplification products were visualised on 2% agarose gels. To authenticate sequence patterns and to exclude potential contamination, at least two PCR products from two independent extractions were obtained.

Table 2
Primer sequences for mitochondrial haplotyping, and amplification strategy. Target lengths are given excluding primers. Reference sequence from Xu and Arnason (1994).

Name	Sequence 5′-3′	Position of target in reference sequence NC_001640	Annealing temperature (°C)	Length of target (bp, colour)
Ec1_f Ec1_r	TTCTTCCCCTAAACGACAACA GACGTACATAGGCCATTCATAAGA	15492-15563	52	72 (green)
Ec4_f Ec4_r	GAATGGCCTATGTACGTCGTG GACTTGGATGGGGTATGCAC	15591-15669	55	79 (4f+4r, pink)
Ec4b_r	ATATTATGTACATGCTTATTATTCA	15591-15609	50	19 (4f+4br, red)
Ec2_f Ec2_r	ACATAACACCATACCCACCTGACA GATGGGGTATGCACGATCAATAAT	15557-15659	55	103 (violet)
Ec5_f Ec5_r	ACCCCATCCAAGTCAAATCA TAGTTGGGAGGGTTGCTG	15696-15758	55	63 (5f+5r, light blue)
Eca1_r	GGCTTGGTGATTAAGCTCGT	15696-15730	52	35 (5f+a1r, blue)

PCR products of mtDNA amplifications were cloned with the TOPO TA Cloning Kit (Invitrogen, Zug, Switzerland) following the manufacturer's protocol, except that the reaction volume was halved. Two clones of each PCR product were Sanger sequenced by Microsynth (Balgach, Switzerland). The products of the ncDNA PCR were premixed with tailed sequencing primers (Binladen et al., 2007) and also Sanger sequenced by Microsynth.

The coat colour SNPs were amplified in multiplex PCR; the first step in 20 μl volumes containing 2.5 U AmpliTaq Gold, 1× GeneAmp 10× Gold Buffer, and 4 mM $MgCl_2$, 0.1 μM of each primer, 20 μg/μl BSA, and 4 μl template DNA. After 30 cycles of amplification as described above, PCR products were diluted 1:20; 5 μl were used in the second, singleplex amplification step (40 cycles) including 0.5 U AmpliTaq Gold, 1× GeneAmp 10× Gold Buffer, and 4 mM $MgCl_2$, 0.2 μM of each primer, and 20 μg/μl BSA. Again, non-template controls were performed and PCR products visualised on 2% agarose gels.

Pyrosequencing of coat colour SNPs of the Basel-GF and Aventicum samples was performed in the Institute for Animal Sciences, Humboldt University Berlin and of Mormont specimens at the Life Science Training Facilities in the Biozentrum, University of Basel according to manufacturer's instructions and Ludwig et al. (2009) on PSQ™ 96MA (Biotage, Uppsala, Sweden).

2.5. Mitochondrial haplotyping

To investigate the matrilineages six partially overlapping and interspersed targets of the mtDNA d-loop with different lengths covering nucleotide positions 15,492–15,669 and 15,696–15,758 (Table 2) (Xu and Arnason, 1994) were amplified. Primers were taken and/or modified from Weber (2005), Cieslak et al. (2010) and Elsner et al. (2014) except for Ec4b_r (this study).

2.6. Molecular sex identification

For the molecular sex identification primer sets Ecab_AMELX/Y from Lippold et al. (2011a) and ZFX/Y_Equus (this study, Table 3) were applied.

Table 3
Primer sequences to identify the molecular sex of horse remains. Reference sequences from Han et al. (2010).

Name	Sequence 5′-3′	Reference sequence	Position of target in reference sequence	Annealing temperature (°C)	Length of target (bp)
ZFX_Equus_F	ATTATATCTGGCCCAGGACT	DQ179230	326–382	55	58
ZFX_Equus_R	TGCCTAGCTTCCAAATCTAA				
ZFY_Equus_F	GAATTTCCTACATGCCCATA	DQ179229	214–263	55	50
ZFY_Equus_R	ATAAAGTCATGAGCCGGATA				

2.7. Coat colour analysis

The investigation of nuclear SNPs in the genes associated with coat colour phenotypes was performed as described in Ludwig et al. (2009). Samples were tested for the basic colours bay, chestnut and black via the *MC1R*- and *ASIP*-loci (Marklund et al., 1996; Rieder et al., 2001). Epistatic dilutions Cream (Mariat et al., 2003) and Silver (Reissmann et al., 2007) and spottings Overo (Santschi et al., 1998), Tobiano (Brooks et al., 2002) and Sabino (Brooks and Bailey, 2005) were examined. In addition, the grey-locus, resulting in a complete white coat in adult horses regardless of original colour, was investigated. This phenotype is caused by a duplication in intron 6 of the *STX17* gene (Rosengren Pielberg et al., 2008). We designed primer pairs to target short fragments of c. 100 bp, first, for the region that spans the end and the beginning of the 4.6 kb duplication (Table 4, red) to detect the mutated allele as well as second, a primer pair to target the end of the fragment (Table 4, blue) which would be present in both wildtype (not grey) and mutated genotype (grey). The suitability of the primer systems was tested on five randomly chosen Freiberger (bay or chestnut) and Camargue (grey) horses each (Fig. S1) at the Institute of Genetics, University of Bern.

2.8. Sequence data analysis

D-loop and *STX17* sequences were examined with BioEdit (Hall, 1999). Mitochondrial haplotypes were determined by comparison with the reference sequence NC_001640 (Xu and Arnason, 1994). We used the nomenclature of Cieslak et al. (2010) to determine the haplotype (Table S2). Haplotype and nucleotide diversity as well as pairwise difference F_{ST} values were computed with Arlequin 3.5 (Excoffier and Lischer, 2010), excluding five ancient samples with missing data (see Table 5). The comparison of Iron Age and modern sequences was done on the basis of haplotype frequencies and pairwise differences for each breed, geographic origin and type (horse, pony). Sequences were pruned to positions 15,494–15,740 to include as many samples as possible. F_{ST} values were interpreted as follows: values from 0 to 0.05 indicate non to little genetic differentiation, 0.05–0.15 moderate differentiation, 0.15–0.25 large differentiation, and values above 0.25 very large differentiation. Negative F_{ST} values equate to zero (Hartl and Clark, 2007: 288). Based on relative haplotype frequencies, Principal Component Analysis (PCA) was computed with PAST (Hammer et al., 2001). Singleton haplotypes were removed from this analysis. Median Joining Networks (MJN) (Bandelt et al., 1999) were constructed with Network 4.6.1.2 (fluxus-engeneering.com) using the following parameters: insertions and deletions were double weighted (default 50), transition: transversion ratio was set to 1:10, and variable sites were down weighted according to the number of deviations from the majority at each nucleotide position excluding haplotype defining sites (Tables S2 & S3). The dataset was reduced to 360 modern (one representative per haplotype per breed) and 14 ancient sequences. The probabilities P of allelic dropout for the SNP loci were calculated as in Gagneux et al. (1997): $P = K * (K/2)^{n-1}$, where K is the number of observed allelic dropouts divided by the number of all positive amplifications of heterozygous individuals, and n is the number of PCR replications.

Table 4
Primer sequences to detect i) the duplication causing premature greying (grey-locus) and ii) the wildtype (no duplication). Figure shows position of target sequences. Reference sequences from Rosengren Pielberg et al. (2008).

Name	Sequence 5'-3'	Position of targetin reference sequence EU606026	EU606027	Annealing temperature (°C)	Length of target (bp, colour)
Both_f	GCACCACCTGGGAACTCA				
Both_r	CTGGAGTGTGCACCAGAATC	5830-5900	10407-10477	52	71 (blue)
Dup_r	GAGAAGTTGGGCAAGAGCAG	-	5830-5889	58	61 (red)

3. Results

3.1. Metrics

The measurements of SD and GL as well as the estimation of withers height of the La Tène and Roman time horses revealed variation at all archaeological sites. Furthermore, based on metacarpi the individuals cluster into a smaller and into a larger group, based on the radii into three groups (Fig. 2). Note that not all individuals are represented by both skeletal elements. There is a linear correlation between length and breadth ($R^2 = 0.81$ for metacarpi, $R^2 = 0.9$ for radii) indicating isometric growth and no robustness differences.

The archaeozoological defined standard withers height of Celtic horses ranges between c. 110 cm to 130 cm (Peters, 1998; Arbogast et al., 2002); the majority of animals investigated here accord to this (Fig. 3). Five horses were larger than 130 cm: three from the Late Iron

Table 5
Differences to the reference sequence NC_001640 between nucleotide positions 15,492–15,669 and 15,696–15,758. Samples indicated by an asterisk have missing data at nucleotide positions: MO10, MO14, MO18: 15,564–15,669; BGF2, AV3: 15,564–15,690 and 15,610–15,669. Cieslak et al. (2010) disregard hotspot nucleotide positions 15,585; 15,597; 15,604 and 15,650 for haplotype assignment, they are mentioned here for completeness.

Sample	Differences to NC_001640 (Xu and Arnason, 1994) 15,…	Haplotype after Cieslak et al. (2010)
MO1	495C; 538G; 602T; 650G; 709T; 720A	I
MO2	495C; 650G; 666A; 720A	D3
MO4	495C	D2
MO5	494C; 495C; 496G; 534T; 585A; 603C; 649G; 720A	X2b
MO6	495C; 602T; 617C; 659C; 720A	B1
MO9	495C; 542T; 585A; 597G; 602T; 615G; 635T; 650G; 666A; 703C; 720A	X3c1 + 615
MO10*	495C; 720A	Haplogroup A/B/C/D/F?
MO12	495C; 585A; 601C; 602T; 720A	F
MO14*	495C; 546T; 703C; 720A	Haplogroup K?
MO15	494C; 495C; 496G; 534T; 603C; 649G; 720A	X2b
MO16	495C; 585A; 601C; 602T; 720A	F
MO17	494C; 495C; 496G; 534T; 603C; 649G; 720A	X2b
MO18*	494C; 495C; 496G; 534T; 720A	Haplogroup X2
BGF1	495C; 585A; 601C; 602T; 720A	F
BGF2*	494C; 495C; 496G; 534T; 603C; 649N; 720A	X2b
BGF3	495C; 542T; 597G; 602T; 631G; 635T; 650G; 666A; 703C; 720A	X3c1 + 631
BGF5	495C; 585A; 601C; 602T; 720A	F
AV2	495C; 542T; 585A; 597G; 602T; 615G; 635T; 650G; 666N; 703C; 720A	X3c1 + 615
AV3*	494C; 495C; 496G; 534T; 602T; 603C; 604A; 720A	Haplogroup X2

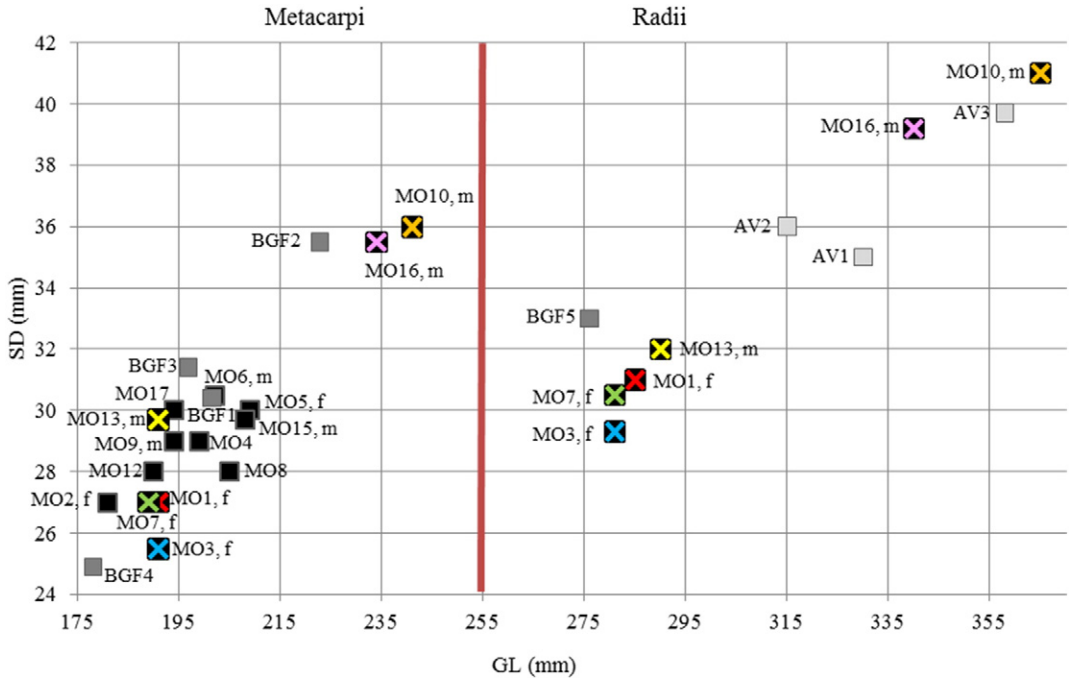

Fig. 2. Greatest length (GL) against smallest breadth of diaphysis (SD) of metacarpi (left panel) and radii (right panel). Bones from Mormont (black), Basel-GF (grey) and Aventicum (light grey). Mormont individuals with data from both skeletal elements indicated by symbol; morphological and/or molecular sex determination: f = female, m = male.

Age: two from Mormont (MO10: 149 cm, MO16: 142 cm) and one from Basel-GF (BGF2: 136 cm), and both Roman time horses (AV1: 136 cm, AV3: 147 cm) from Aventicum. The average withers height of the Mormont horses was, based on 15 measurable metacarpi and six radii, 124 cm (119 cm without the two large animals). The average in Basel-GF was 120 cm and in Aventicum 137 cm (141 cm for the Roman time individuals).

3.2. Mitochondrial haplotyping

In Mormont ten metacarpi were genetically well preserved, three only allowed for a partial amplification of the target region, and five had no amplifiable mtDNA preserved. It was possible to amplify mtDNA from four out of six samples from Basel-GF and from two out of three radii (one Iron Age, one Roman) from Aventicum. This results in an overall amplification success of 56% (19/34).

We identified eleven haplotypes belonging to six haplogroups (hg). Most prevalent is hg X2 (n = 6), followed by hg F (n = 4), hg X3 (n = 3), hg D (n = 2), hg B (n = 1) and hg I (n = 1). Additionally, one sample each might be associated with hg K and hgs A/B/C/D/F, respectively (Table 5). Haplotype sharing within and between archaeological sites was observed: haplotype F is shared by MO12, MO16, BGF1 and BGF5. MO15 and MO17 have the same haplotype X2b, MO5 and BGF2 also share variants of X2b, and despite some missing data MO18 and AV3 can be assigned to haplogroup X2 as well. MO9, BGF3 and AV2 possess variants of haplotype X3c1, one of which previously undescribed (BGF3). Horses with withers height below 130 cm carried haplotypes B1, D2, D3, F, I, X2b, and X3c1; they share haplotype F and haplogroup X2 with the large horses. Haplotype (0.92 ± 0.06) and nucleotide (0.3 ± 0.02) diversity is very high in the investigated Iron Age horses (Table S4). This result is mirrored by the horses from Mormont: haplotype diversity = 0.97 ± 0.06, nucleotide diversity5 = 0.3 ± 0.02.

3.3. Molecular sex identification

Molecular sex could be identified for five specimens from Mormont, the other samples were not preserved well enough to enable the amplification of targets in the amelogenin and/or zinc-finger genes (Table 6). This confirms the results of the morphological sex determination for MO5 and MO16; MO2, MO6 and MO9 were previously undetermined.

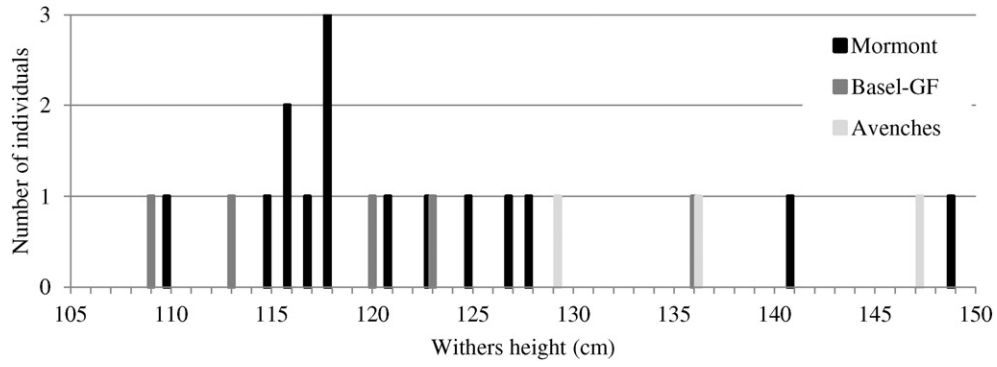

Fig. 3. Estimated withers height (May, 1985) in cm from metacarpi and/or radii from Mormont (black), Basel-GF (grey) and Aventicum (light grey). When measurements of both bones were available, the mean was used.

Table 6
Results for molecular sex identification with *AMELX/Y* and *ZFX/Y*.

Sample	AMELX	AMELY	ZFX	ZFY	Sex
MO2	1/3	0/3	1/2	0/3	Female
MO5	1/3	0/2	2/2	0/4	Female
MO6	0/2	1/2	2/2	1/3	Male
MO9	0/2	1/1	2/2	2/3	Male
MO16	0/1	0/1	2/2	2/2	Male

3.4. Coat colour

For six of the Mormont and two of the Basel-GF samples DNA preservation allowed for the amplification of SNPs in genes associated with coat colour (Table 7). The specimens from Aventicum were not preserved well enough for SNP typing. All positive samples revealed basic colourations. One large horse (MO16) was bay, of the smaller horses (<130 cm) three had a black coat and four were chestnut. We did not detect any mutations in the dilution and spotting loci. The Mormont specimens were also tested for the duplication that causes premature greying. For five individuals the target at the end of the 4.6 kb fragment present in both wildtype and mutated genotype could be amplified twice (once for MO6, see Table 7), but the shorter PCR products for the grey-locus were negative four times for each individual suggesting all tested animals were not grey. The probability of allelic dropout for the only heterozygous SNP, *MC1R*, was $p = 0.0015$ with four PCR replications.

3.5. Comparison with modern horses

The Median Joining Network shows the widespread distribution of Late Iron Age horse mtDNA d-loop sequences within the variation of southern European horse and European pony breeds (Fig. 4). The ancient horses are represented in the major haplogroups (B, D, F, I, X2, X3) except for hg K (see above).

The fixation index based on the number of pairwise differences between each breed and the ancient horses indicated substantial structuring ($F_{ST} = 0.12$, $p < 0.001$) (see Table S5 for pairwise population F_{ST}). The genetic differentiation between the geographic regions is moderate ($F_{ST} = 0.06$, $p < 0.001$). F_{ST}-values for the regional groups indicate close relationships between all European horse and pony breeds and the Celtic horses, except for the Spanish breeds which are moderately different (Table S6). This outcome is supported by PCA based on the frequencies of haplotypes with loadings >10% (Fig. 5; frequencies of all haplotypes in Fig. S2). The Iron Age horses stand out from the modern breeds because of their unique variability but tend to be closer to the northern European ponies which share all haplotypes present in the ancient dataset.

4. Discussion

This study provides first insight into mtDNA d-loop diversity, molecular sex and coat colour of Late La Tène horses in Switzerland. DNA preservation was as expected for the region and time frame explored, however, the results for the nuclear markers were better for the recently excavated site on the hill of Mormont (Pruvost et al., 2007; Bollongino et al., 2008). The withers height of the investigated horses fell mostly within the variation of standard Iron Age horses, yet five individuals were larger than 130 cm reaching up to 149 cm. Two of these were identified as male, but smaller stallions were also present, thus sexual dimorphism does not explain the height differences. Moreover, the relation between length and breadth (robustness) is linear, indicating that smaller and larger horses did not belong to different morphotypes (Brooks et al., 2010; Dzierzecka and Komosa, 2013). Only one horse exceeded the defined pony range of maximal 148 cm. The investigation of the maternal lineages revealed high nucleotide and haplotype diversity within the upper range of variance in modern European breeds (see Table S4). The horses belonged to six haplogroups (B, D, F, I, X2, X3) which are rare in wild horses (Weinstock et al., 2005; Cieslak et al., 2010; Lorenzen et al., 2011; Orlando et al., 2013). They were first described from domestic horses from sites in Novosibirsk, Russia, dating to c. 2000 BCE, and Moldova and Romania around 1250 BCE (Cieslak et al., 2010). A haplogroup-package dominated by hgs D, K, X2 and X3 is subsequently found in archaeologic sites throughout Eurasia (Vila et al., 2001; Di Bernardo et al., 2004; Keyser-Tracqui et al., 2005; McGahern et al., 2006; Cai et al., 2007; Cai et al., 2009; Lira et al., 2010; Cieslak et al., 2010; Priskin et al., 2010; Bower et al., 2013).

The large matrilineage diversity is striking, particularly given that the majority of horses, namely those from Mormont, belonged to a potentially contemporaneous local population which should include directly related individuals. The occurrence of shared lineages in the sites Mormont and Basel-GF might indicate horse exchange between Helvetii and Rauricii. The ancient matrilineages show affinities to modern horse and pony breeds from most regions investigated. They tend to have bequeathed more to northern European and British Isles pony breeds and less to Spanish breeds. However, considering that domestic horses were first introduced to Switzerland in the Late Neolithic or Early Bronze Age, i.e. around 2500–2000 BCE (Schibler and Studer, 1998: 177), it is remarkable that most major haplogroups are present in the Iron Age archaeological record, notably in view of the rareness of archaeologic horse remains.

With morphological and molecular methods, five mares and seven stallions were identified, all from the hill site of Mormont. This corresponds to the male:female ratio of 1:1.5 amongst all morphologically sex determined horses in Mormont (Méniel, 2014: 17).

Coat colouration seems to be rather uniform. We would expect more variety: Ludwig et al. (2009) documented a rapid and substantial increase in coat colourations already in the Bronze Age, at least in Asia

Table 7
Results from the coat colour analysis. In addition to the phenotype, the genotypes for the eight SNPs investigated are shown; differences from the wildtype are indicated in bold and only detected at the *ASIP*- and *MCR1*-loci. The duplication causing premature greying was not detected in any of the Mormont samples (Avenches and Basel-GF samples were not tested). Unreproducible results are parenthesised.

Sample	Phenotype	Nuclear Genes								
		ASIP	*MC1R*	*EDNRB* Overo spotting	*KIT13* Tobiano spotting	*KIT16* Sabino spotting	*MATP* Cream dilution	*SILV9* Silver dilution	*SILV11* Silver dilution	GREY
MO4	(Chestnut)	**(a/a)**	**(e/e)**	ov/ov	KM0/KM0	(sb1/sb1)	C/C	(z/z)	z/z	–
MO5	Black	**a/a**	**E/e**	ov/ov	KM0/KM0	sb1/sb1	C/C	z/z	z/z	No
MO6	Black	**a/a**	**E/e**	ov/ov	KM0/KM0	sb1/sb1	C/C	z/z	z/z	(No)
MO9	Black	**a/a**	**E/e**	ov/ov	KM0/KM0	sb1/sb1	C/C	z/z	z/z	No
MO16	Bay	A/A	E/E	ov/ov	KM0/KM0	sb1/sb1	C/C	z/z	z/z	No
MO17	Chestnut	**a/a**	**e/e**	ov/ov	KM0/KM0	sb1/sb1	C/C	z/z	(z/z)	No
BGF1	Chestnut	**a/a**	**e/e**	ov/ov	KM0/KM0	sb1/sb1	C/C	(z/z)	z/z	–
BGF4	Chestnut	–	**e/e**	–	KM0/KM0	–	C/C	z/z	z/z	–

Differences from the wildtype are indicated in bold; the wildtype is given in capital letters in the genetic nomenclature.

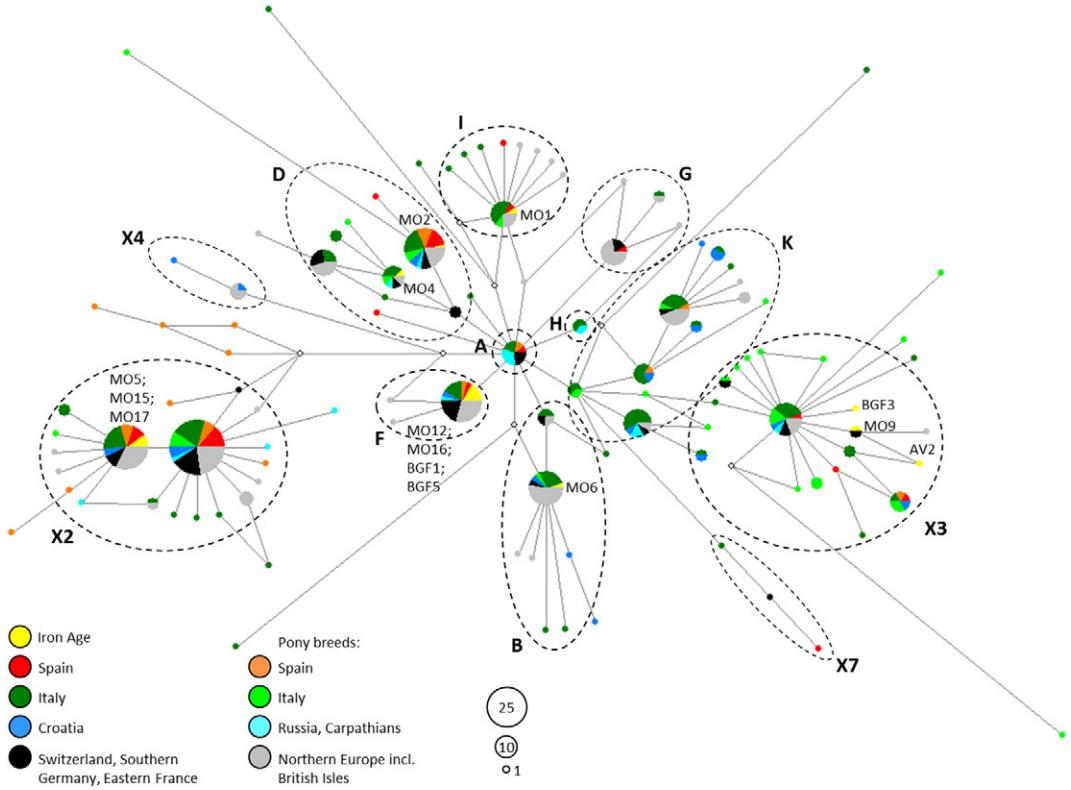

Fig. 4. Median Joining Network of 14 ancient and 355 modern mtDNA d-loop sequences. Circle size is proportional to haplotype frequency. Nomenclature follows Cieslak et al. (2010).

and eastern Europe, and in northern Europe spotted Iron Age horses were detected (Svensson et al., 2012) in comparable datasets. However, monochrome horses were favoured over skewbalds and white markings on face and leg according to written Roman sources (e. g. Corp. Hippiatr. Gr., 1, 115: Oder and Hoppe, 1924–1927; Geop. 16,2: Wappmann, 1985). Breeders probably had noticed hearing, visual, and neurological impairment and even stillbirths coming along predominantly with spottings, especially in homozygous individuals

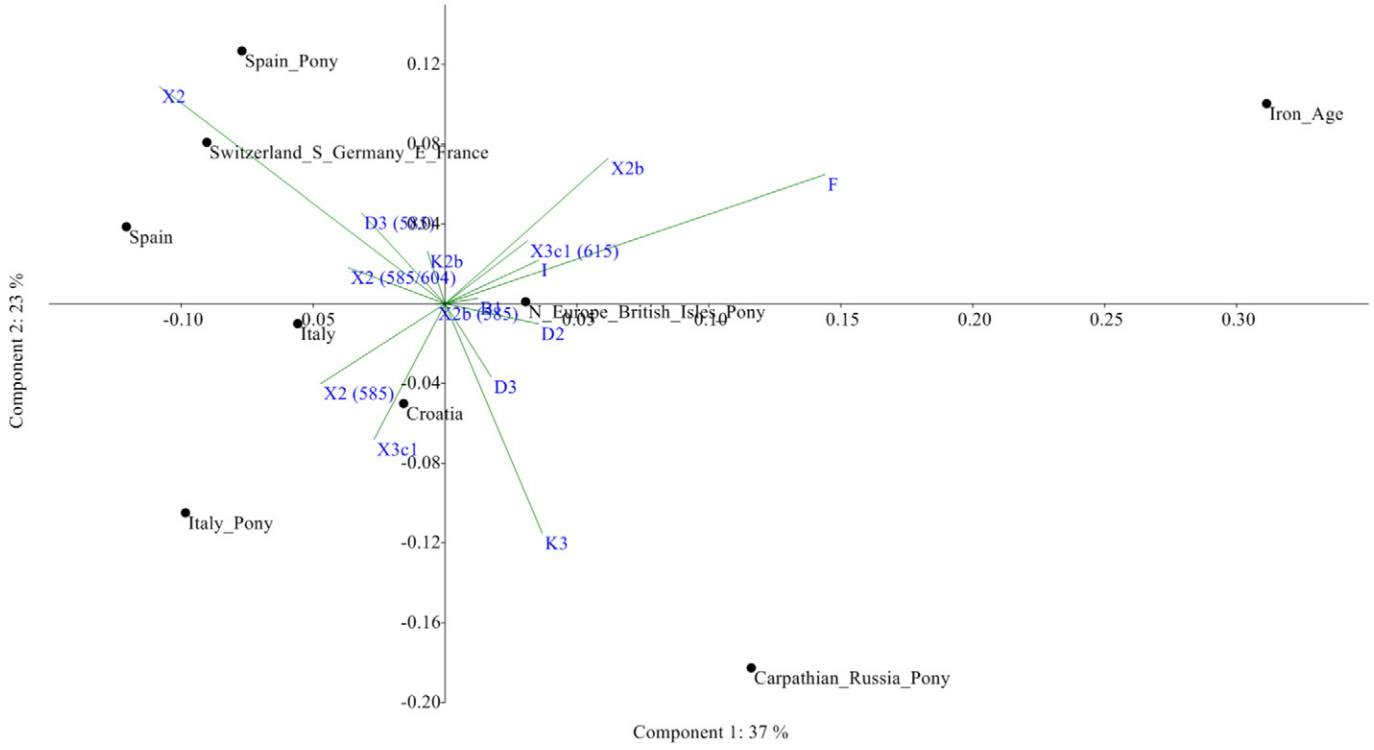

Fig. 5. PCA plot based on relative mtDNA haplotype frequencies of the haplotypes present in the Iron Age horses and additional haplotypes with loadings over 10% (see Fig. S2). The two dimensions display 60% of the total variance.

(Reissmann, 2009: 20; Bellone, 2010) and thus may have selected against those phenotypes. Notably premature greying resulting in a white coat regardless of original colour was also not detected. Again, we expected a different result, as white horses played an important role in Roman and also in Germanic ceremonies (Tacitus, Germ. 1,10: Kretschmer, 1986; Johnstone, 2004: 61). However, of the horses investigated, only the specimens from Aventicum stem from an unambiguous ritual context and those were not preserved well enough for genetic coat colour identification. Assuming that ceremonial activities were also performed on the hill of Mormont, we find no evidence that specially coloured horses were preferred in ritual, which apparently also applies for sex as both females and males were present.

Some important archaeozoological questions concerning horse exchange, breeding aims and selection criteria e.g. for ceremony have to remain open yet, due to both the small sample size and insufficient data for comparison. Further complicating is the fact that Roman horses, too, were not standardised and the workaday animals were usually smaller than military or circus breedings (Junkelmann, 1990: 39). It appears that mtDNA d-loop variation offers too little structuring, but because it is highly conservative the investigation of the paternal inherited Y-chromosome cannot contribute to the question of horse provenience either (Lippold et al., 2011a; Wallner et al., 2013). The exploration of nuclear markers beyond sex and colouration (Schubert et al., 2014) might give insight into possibly distinct characteristics of horses bred by Celtic and Roman stud farmers. Further approaches like stable isotope analysis, particularly of strontium (Slovak and Paytan, 2011), seem even more promising to solve these important issues. It might thus be necessary to dig deeper into the past and to include archaeological material from both Rome and its provinces, and regions outside the Roman Empire to trace the origin and breeding history of small and large horses.

5. Conclusion

The horse remains excavated from the archaeological sites Mormont, Basel-GF and Aventicum, dating to 150–50 BCE (La Tène D) and to c. 100 CE, revealed a high diversity of matrilineages. The most frequent haplogroups F and X2 were shared between the sites, and between small and large horses. While all determinable large individuals (>140 cm withers height) were male, both sexes were present amongst horses with withers heights between 110 and 130 cm. The detection of solely monochrome coats and the absence of white colourations might indicate selection. The simplified approach to detect premature greying developed in this study provides a relevant tool for aDNA research concerning the phenotypes of (pre)historic horses. Compared to modern indigenous breeds, Late Iron Age horses seem to have bequeathed more to northern European ponies than to Spanish breeds. By broadening the methodological spectrum and by complementing the dataset both spatially and temporally, we hope to address archaeozoological questions on horse exchange, breeding aims, and selection criteria e.g. in ritual contexts more comprehensively.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jasrep.2016.03.007.

Acknowledgements

Samples were kindly provided by Archéologie Vaudoise (Mormont), Site et Musée Romain d'Avenches (Aventicum) and Archäologische Bodenforschung Basel-Stadt (Basel-Gasfabrik). The authors thank Tosso Leeb and Michaela Drögemüller (Institute for Genetics, University of Bern), Andreas Papassotiropoulos and Kim Dung Huynh (Biozentrum, University of Basel), Arne Ludwig (Institute for Zoo- and Wildlife Research) and Mélanie Pruvost (then Institute for Animal Sciences, Humboldt University Berlin), Patrice Méniel and Pauline Nuviala (University of Burgundy, Dijon), co-editor Andy Howard, University of Durham, and one anonymous reviewer. Julia Elsner was funded by Swiss National Science Foundation (K-31K1_120528/1), Freiwillige Akademische Gesellschaft Basel, Nikolaus und Bertha Burckhardt-Bürgin-Foundation.

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