

Setúbal Arqueológica
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O CASTRO DE CHIBANES NA CONQUISTA ROMANA

Intervenções arqueológicas de 1996 a 2017



**O CASTRO DE CHIBANES
NA CONQUISTA ROMANA**

Intervenções arqueológicas de 1996 a 2017

Coordenação
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Genomic analysis of cattle from the Roman Republican fortification of Chibanes, Palmela, Portugal

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Introduction

Many different perspectives are already documented about how and what did the Roman Empire change in its extensively conquered territories. It is undeniable that the Romans had an enormous impact all over Europe, and this can be observed to this day, as they shaped fundamental characteristics of the regions they occupied, including the language (Lloyd, 1989; Stehling, 2013). In this study we will investigate one aspect of the Roman way of life: their cattle.

This study aims to understand the relationship between cattle raised by the Romans in a fortification from the 1st and 2nd century BC, i.e. the Roman Republican period, and their extant counterparts. The fortification in focus is Castro de Chibanes, a settlement located in Palmela, Portugal. This report will introduce background information about the

Early Roman occupation in Europe, the faunal assemblages from this period and how cattle changed (based on osteometry), as well as the relevance of archaeogenomic data to give further insight to questions that zooarchaeological work cannot answer. It will also provide an overview of the molecular methods used in the analysis of cattle remains from Chibanes. These data will be interpreted in complement to the osteometric information available for cattle from Roman times in the Iberian Peninsula. This study will present the results from both the archaeogenomic and zooarchaeological approaches in an attempt to answer questions such as if cattle were improved by the Romans, and if new stock carrying specific maternal lineages were introduced in this site.

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ZOOARCHAEOLOGICAL CONTEXT

The Roman Empire is known to have been one of the greatest empires in the history of humankind. During its prime, it dominated areas in three different continents. It was well organized and efficient in connecting its many territories, maintaining communications and trade between them, and enlarging the economy, which made it so successful (Soto, 2013).

Archaeological findings suggest that, since the Punic Wars in the 3rd - 2nd centuries BC, contacts between the Iberian Peninsula and the Roman Empire increased. The occupation became well-established during the second half of the 2nd century BC as Lusitania became part of the province of *Hispania Ulterior*. In 27 BC *Caesar Augustus* turns it into the province *Lusitania et Vettones*, and names its capital *Emerita Augusta*, today Mérida (Valenzuela-Lamas and Detry, 2017).

The study of faunal remains is highly relevant: new genomic tools combined with zooarchaeological information are starting to yield valuable data about the domesticated animals raised by the Romans and on how the trade of livestock occurred during the Roman period (Colominas and Edwards, 2017).

FAUNAL ASSEMBLAGES FROM ROMAN CONTEXTS

With the military advances and the creation of settlements and commercial routes during the Roman period, changes in animal consumption in the conquered areas probably occurred, which relates to differences in the types of animals that were exploited. In the historical sites, zooarchaeologists characterize the frequencies of the different livestock species found, which gives important information on which species were more numerous in those settlements and about their exploitation.

From the different faunal assemblages characterized, dated to the Roman period, there are species that are common across the different areas of the Empire, including: domesticated animals, such as sheep, cattle and pigs; smaller and bigger game, such as deer, rabbits and birds; and seafood (Ureña *et al.*, 2021; Detry, Tavares da Silva and Soares, 2017).

During the first millennium BC, before the Roman conquest, cattle frequencies decreased in different parts of the Empire, such as Denmark (Wells and Randsborg, 1993), Britain (Hambleton, 1999; Albarella, 2007), and France (Columeau, 2003). As for Italy, a decrease in cattle frequencies was also observed between the Middle (17th - 14th century BC) and the Final Bronze Age (1150-950 BC).

In contrast, during the Roman occupation in the Western Roman Empire, e.g. Roman Britain, Germany and Gaul (King, 1984) (but also in other places in the Western Mediterranean, such as Mallorca (Valenzuela *et al.*, 2013), there was a generalized increase of both cattle and pig remains. Some explanations revolve around the cultural and political differences that were introduced by the Romans, as they probably had different requirements for cattle production and diet preferences (Albarella, 2007; King, 1999). Although there was a general increase, the case was not the same everywhere, as in Italy there was still a higher frequency of pig remains, which is not observed for the rest of the Empire. This probably means that different ways of life co-existed during Roman times. In fact, King (1984) proposed that the “cattle pattern” observed for Britain was characteristic of Germany and northern Gaul.

As for regions such as southern Gaul and the Iberian Peninsula, the influence came mainly from Italy. In some places of Iberia, specifically in Roman Spain (Colominas, 2013; Eres, 2017), the faunal assemblages reveal that pigs were more numerous than other domestic species. This contrasts with other Iberian sites where goats, sheep and cattle continued to be more frequent, probably related with changes in local production since cattle represents a small proportion of Italian Republican faunal assemblages (Valenzuela-Lamas and Albarella, 2017).

As for present-day Portugal, there were no significant changes as it seems the Romans adapted to what was already available in the area, that is: domesticated animals such as sheep; wild species, such as deer; or molluscs in sites like Chibanes closer to aquatic ecosystems (Davis, 2006; Detry, Tavares da Silva and Soares, 2017; Valenzuela-Lamas and Fabião, 2012).

OSTEOMETRIC STUDIES OF ROMAN CATTLE

Did animal husbandry practices change under the Roman influence? Did the Romans try to improve cattle available in the regions they occupied or introduced new stock? To answer these questions, zooarchaeologists use a way of comparing cattle from different periods across time, i.e. osteometric measurements. These measurements characterize bone sizes (e.g. lengths and widths), and shape. It allows to determine differences in cattle size and also other information, such as frequency of males *vs* females.

Larger body sizes can be seen as a desirable characteristic as it is usually associated with more labour

force and more meat. Yet, larger animals might not adapt to harsher climates and situations, as they require more resources for their maintenance (Valenzuela-Lamas and Albarella, 2017). For example, in some places, an increase in the size of cattle was observed during Roman times: in Great Britain, several different sites revealed larger cattle sizes (Dobney, Jacques and Irving, 1996); in the Netherlands, there were cattle of two different size groups (Lauwerier, 1988); and in Germany (Teichert, 1984) and France (Lepetz, 1995) a size increase was also observed.

The differences are seen in many regions across the Empire and there are two main hypotheses when trying to explain this phenomenon. The first one is that there was trade of cattle between different regions of the Roman Empire, which implies the import of non-local animals (Dobney, Jacques and Irving, 1996; Lauwerier, 1988; Teichert, 1984). The second hypothesis suggests that the size increase resulted from different animal husbandry techniques being introduced in the newly conquered areas (Forest and Rodet-Belarbi, 2002).

The data are still scarce, but Detry *et al.* (2021), discuss preliminary results on how the size improvement varied during this period given, for example, differences in location, importance of the site, previous occupation, etc. To summarize, it is suggested that cattle were larger in newly founded cities such as *Emerita Augusta* (Mérida) rather than in those with indigenous origin, such as Alcáçova de Santarém. There is also the discussion that *villae* could have played a very important role in supplying cities with food, which could signify there was also cattle trade between the cities and the *villae*. The reasons behind the improvement in size are still unknown as many variables could have influenced it, such as the need for bigger animals for agricultural labor, the increase of the population which would lead to a bigger demand for meat, or simply better nourishment of the animals. The location also matters, since settlements closer to the coast would also have allowed fishing, consuming molluscs and various seafood, rather than livestock (Detry *et al.*, 2021).

CATTLE GENETICS

European cattle have their origins in the domestication of the extinct Eurasian aurochs (*Bos primigenius*) around 10,500 years ago in the Near East (Verdugo *et al.*, 2019). The process of aurochs domestication is still poorly understood, but multidisciplinary and

innovative methods are being used so the history of cattle domestication and expansion can be unveiled.

Various genetic markers available, such as mitochondrial, Y-chromosome and whole-genome sequence information, allowed scientists to bring new perspectives to the discussion. For the most part genetic data are consistent with zooarchaeological findings but add detailed information regarding the genetic composition of past animals, the relationships among individuals from different regions, and may even reveal some of their phenotypic traits (for example, biological sex and coat colour).

Mitochondrial DNA has been a useful tool to investigate the origin and expansion of cattle (Achilli *et al.*, 2009). Initial studies were focused on a segment of the hypervariable control region, revealing major haplogroups (e.g. T, T1, T2, T3, T4). As looking to these small parts of the mitogenome is insufficient to understand evolutionary histories (the control region is non-coding and fast-evolving), more comprehensive analyses are needed.

With further studies (e.g. Achilli *et al.*, 2009; Bonfiglio *et al.*, 2010, 2012), it was revealed that: the macro-haplogroup T is divided in two sister haplogroups, T5 and T1, T2 and T3 (T4 ended up being nested within T3). The T1-African haplogroup is also divided in sub-haplogroups from T1a to T1f (Bonfiglio *et al.*, 2012).

Besides the diversity of T haplogroups in taurine cattle, new rare haplogroups were found: one animal from Korea was assigned to the P haplogroup (only observed so far in extinct aurochs specimens) and other animals belong to the distinct Q haplogroup (Achilli *et al.*, 2009; Ginja *et al.*, 2019). Data from the analysis of complete mitogenomes also allowed for a new matriline to be identified in native cattle breeds from Italy: the R haplogroup (Achilli *et al.*, 2009). Other than *Bos taurus* cattle matriline, there is the I matriline typical of zebu cattle (*Bos indicus*) and which is divided in haplogroups I1 and I2 (Utsunomiya *et al.*, 2019).

THE EXPANSION OF CATTLE IN EUROPE

Cattle derive from two domestication events of aurochs that took place in the Near East and the Indus Valley and which originated taurine (*Bos taurus*) and zebu (*Bos indicus*) stocks, respectively. Cattle spread from these primary domestication centers across different continents accompanying the various human migrations (Achilli *et al.*, 2009; Scheu *et al.*, 2015). The diversity of maternal lineages observed in Europe and

Africa is a subset of that from the Near East, the most frequent being T3 and T1, respectively (Lenstra *et al.*, 2014). Verdugo *et al.* (2019) suggest African cattle originated in the Levant (geographical area in the Eastern Mediterranean region of Western Asia, e.g. present-day Israel) and then spread across the north of Africa.

During the Roman period, the contact between the areas surrounding the Mediterranean Sea, i.e. North Africa and Western Europe, is suggested to have been even more intense than in previous periods judging by the diversity of cattle matrilineal groups (Colominas *et al.*, 2015). The presence of T1 cattle was maintained throughout the Roman period in the Iberian Peninsula, as there are records of this haplogroup and also T3 being identified in samples dated to the Roman period in this region (Colominas *et al.*, 2015).

EXTANT IBERIAN CATTLE

The classification of cattle breeds has many different variables and is rather complex. To briefly explain some aspects of breed classification: it can be based on parameters such as geography, morphology, and origin (Feliu *et al.*, 2011). Breeds have been organized by continent, which makes sense since cattle with different continental origins are likely to have differentiated somewhat independently. Breeds are also classified according to regions that vary in climate and other geographical differences. In general, recent molecular data provide support for geographic breed grouping (Ginja *et al.*, 2019). Although these subdivisions are important, morphology and history seem to be more relevant. For such a small geographical area, the Iberian Peninsula has a rather large diversity of breeds. In Portugal only, there are 16 native cattle breeds: Alentejana, Algarvia, Arouquesa, Barrosã, Brava de Lide, Brava dos Açores, Cachena, Garvonesa, Jamelista, Marinhosa, Maronesa, Mertolenga, Minhota, Mirandesa, Preta and Ramo Grande (<https://www.fao.org/dad-is/en/>).

The diversity of these breeds can be studied in different ways using both sex markers, the chromosome X and Y. Both sex chromosomes are used in population genetic studies, as they have the potential to trace historic demographic events (Anderung *et al.*, 2007; Pelayo *et al.*, 2017). Other genome-wide methods, such as whole-genome resequencing, provide additional information on the different lineages and the origin and spread of gene variants (Feliu *et al.*, 2011).

Different factors contribute to these numerous breeds: the relative genetic isolation, the adaptation

to local environments, selection for specific traits and the influence from other European and African cattle (Martín-Burriel *et al.*, 2011). The different breeds are genetically very distinct and show high levels of genetic variation even though despite the relatively small geographical area they are constricted to. The Iberian cattle breeds also seem to conserve much of the “original” diversity with strong influence of African cattle, rather than recent admixture with commercial breeds such as Holstein, Angus, etc. (Da Fonseca *et al.*, 2019; Martín-Burriel *et al.*, 2011).

Materials and Methods

In this section the archaeological context of the specimens under study and the genomic methods used are described. In particular, sub-sampling of the archaeological remains, DNA extraction, preparation of genomic libraries, sequencing and bioinformatic analyses are described as in Ureña *et al.*, 2021 (with some modifications as appropriate).

THE ARCHAEOLOGICAL CONTEXT – OSTEOLOGICAL CHARACTERIZATION OF THE SPECIMENS FROM CHIBANES

Chibanes was the object of archaeological excavations in the beginning of the 20th century by A.I. Marques da Costa, and between the years 1996 to 2017 by Carlos Tavares da Silva and Joaquina Soares (MAEDS-Museu de Arqueologia e Etnografia do Distrito de Setúbal) (Tavares da Silva and Soares, 1997, 2014). The archaeological context has been thoroughly described (Soares *et al.*, 2019).

The Roman Republican occupation in Chibanes is characterized by two phases: the first from ~125-75 BC and the second from ~75-50/40 BC. Of a total of 5594 vertebrate remains retrieved from this site, for the first phase of the Roman Republican occupation, only 93 (1.7%) were cattle. An increase was observed in the second phase, as 342 (3.6%) *Bos* remains were found, which could be due to a more stable occupation (as it is hard to move bigger animals from place to place). From these cattle remains, the archaeologists estimate that the minimal number of animals was 3 for the first phase and 11 for the second.

The 9 bones that provided useful measurements were selected for an osteometric analysis. Three of these remains, representing different individuals, were then used in the archaeogenomic analysis carried out in this study. The contexts of these specimens are presented in Table I.

The osteometric measurements were taken by Cleia Detry and interpreted in the context of other osteometric data (Detry, Tavares da Silva and Soares, 2017). With the aim of depicting the size of cattle from Chibanes in relation to those of other Roman contexts, we elaborated the graphics of Figures 1 and 2. We used RStudio v. 1.4.1106, with packages *zoolog* (<https://josempozo.github.io/zoolog/>) and *ggplot2* (Wickham, 2016). The histograms represent the cattle widths (Figure 1) and lengths (Figure 2), in a logarithmic scale, observed throughout the Roman Period,

i.e. the Roman Republican, Early Roman Empire and Late Roman Empire, as well as the preceding Iron Age times. The archaeological sites considered were the following: Mérida (MER), Alcáçova de Santarém (AS) and Mesas do Castelinho (MC). The data represented corresponds to a ratio obtained from the measures of each sample (from each site) compared to the reference measurements of a small Bronze Age cow (Nieto-Espinet, 2018), making it possible to observe if the samples collected at these different sites represent cattle of larger or smaller body sizes.

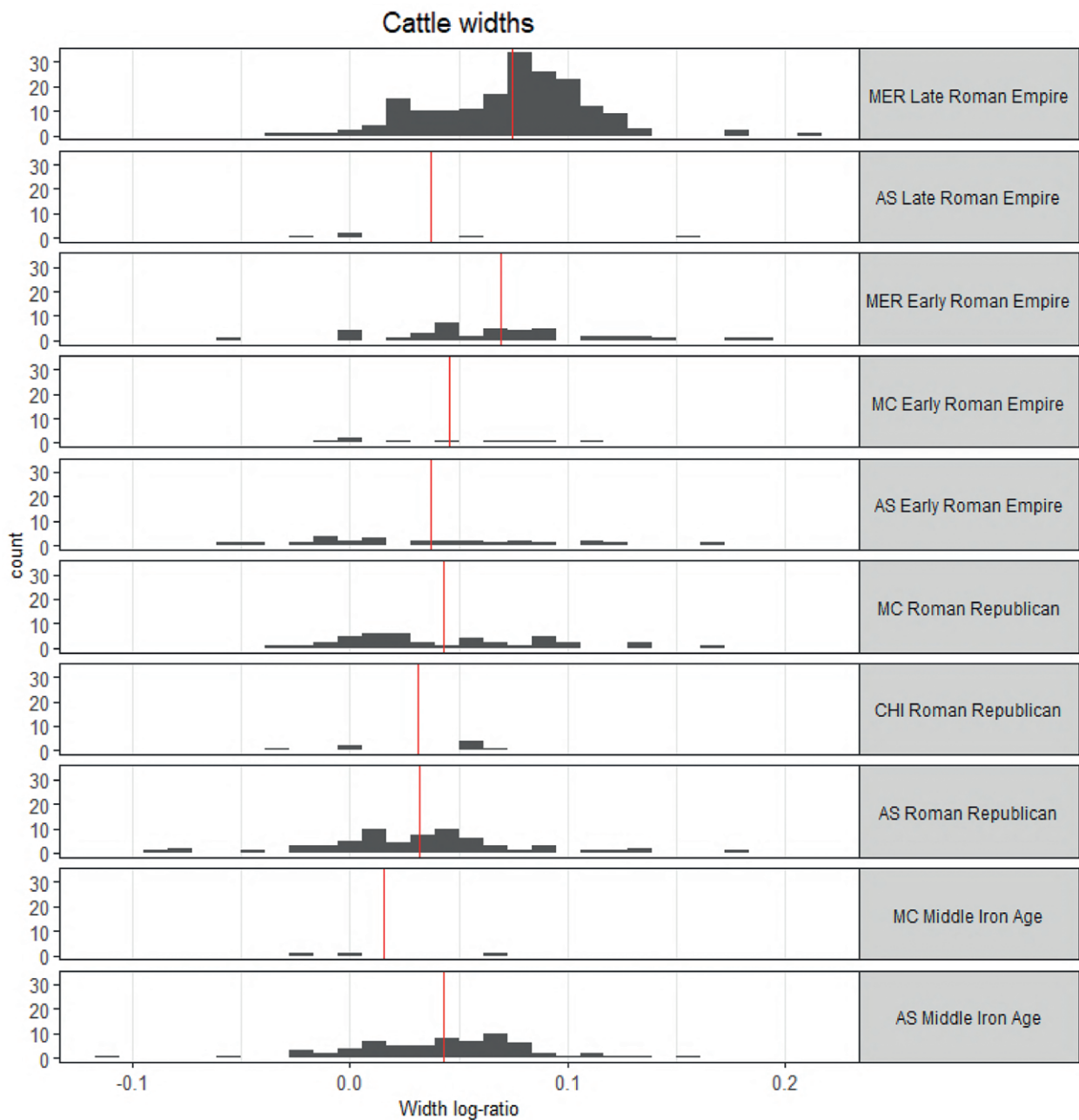


Figure 1 - Histogram showing the width log-ratios of cattle specimens collected at different archaeological sites located in the Iberian Peninsula (MER-Mérida, AS-Alcáçova de Santarém, MC-Mesas do Castelinho, CHI-Chibanes) and representing different historic periods. The red line represents the mean value for each context.

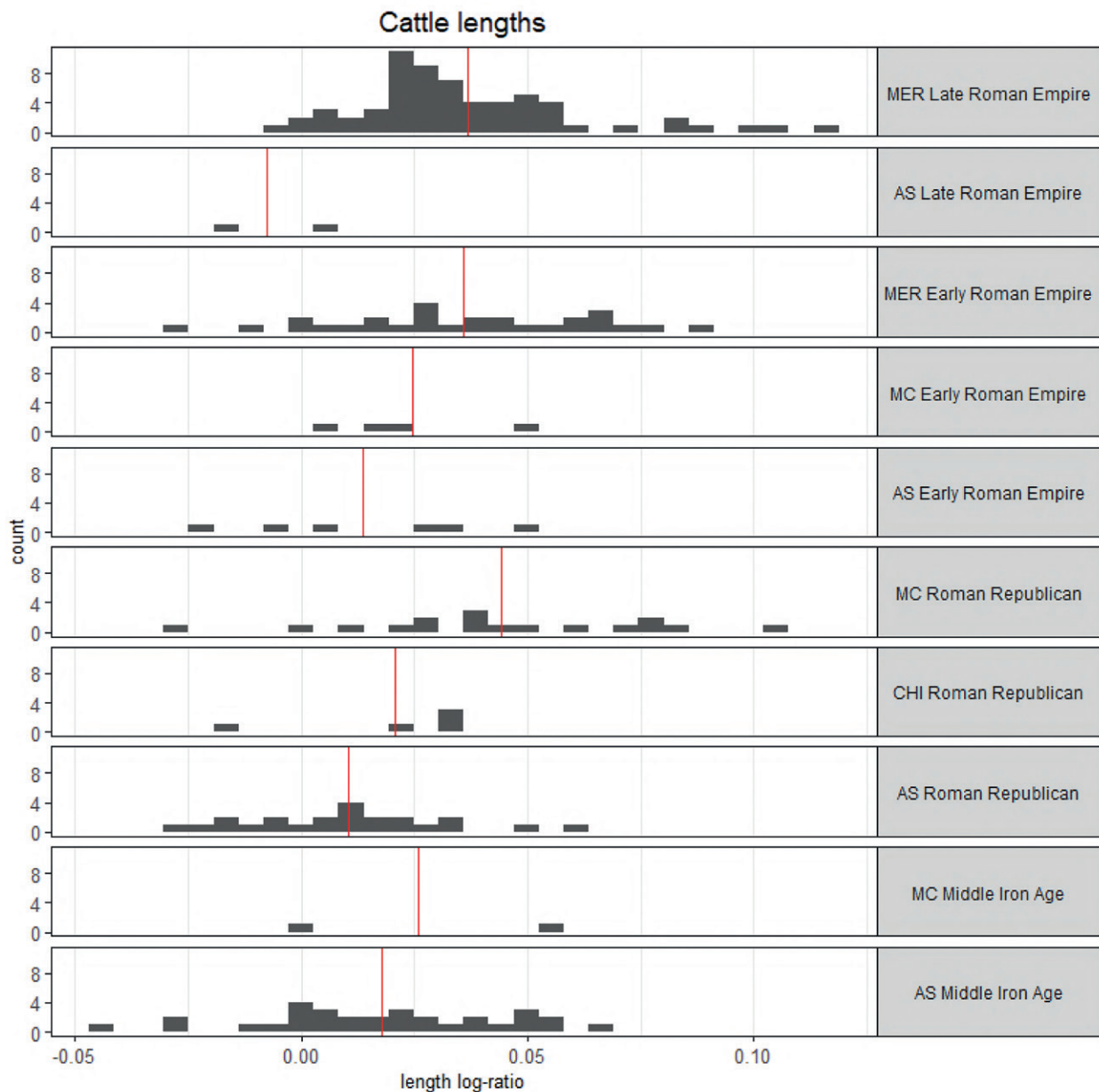


Figure 2 - Histogram showing the length log-ratios of cattle specimens collected at different archaeological sites located in the Iberian Peninsula (MER-Mérida, AS-Alcáçova de Santarém, MC-Mesas do Castelinho,CHI-Chibanes) and representing different historic periods. The red line represents the mean value for each context.

By comparing the cattle materials from Chibanes with those from other sites, ranging from the Middle Iron Age to the Late Roman Empire, despite the low number of materials available we can observe that:

- During the Roman Republican Period, the mean widths observed in Chibanes are very similar to those of Alcáçova de Santarém and slightly smaller than those observed for Mesas do Castelinho.

- The mean values of the lengths, Chibanes cattle appear to be slightly higher than those of Alcáçova de Santarém but still smaller than what was observed for Mesas do Castelinho.

In the overall context, across the different periods of the Roman occupation, Chibanes' specimens are considerably smaller to those of Mérida from later periods, but very similar in widths and longer than what is observed in Alcáçova de Santarém even in the later stage of the Roman Period.

ANCIENT DNA LABORATORY PROCEDURES SUB-SAMPLING OF ARCHAEOLOGICAL REMAINS

The analyses were carried out in dedicated ancient DNA (aDNA) facilities at the Centre for

Table 1 - Information about the bones used in the ancient DNA analyses.

Sample ID	Scientific name / Common name	Type of material	Location (in the site)	Chronology
CHI004	<i>Bos taurus</i> /cattle	Metatarsal	Sector IV; Squares K11-K12; Layer 3; Fieldwork 1996	2nd -1st century BC Phase IIIA
CHI008	<i>Bos taurus</i> /cattle	Molar (M3)	S. IV; Locus L12; Squares H-I-J/10-11; Layer 3; Fieldwork 1997	2nd -1st century BC Phase IIIA
CHI009	<i>Bos taurus</i> /cattle	Mandible with M3	S. IV; Squares L-M/10-11; Layer 3; Fieldwork 1996	2nd -1st century BC Phase IIIA

Palaeogenetics, Stockholm University, using strict procedures to prevent contamination.

The sub-samples were collected from three different remains: a metatarsal and two inferior molar teeth (M3).

The specimens were exposed to UV radiation for 10 minutes on each side to remove potential surface contaminants. Following, the external surface in target areas was removed with a sterile blade. We obtained cc. 80 mg of bone powder from each sample using a multitools drill (Dremel™) at low speed to avoid local heating.

DNA EXTRACTION AND PURIFICATION

A guanidinium/silica-binding method was used for DNA extraction optimized for short DNA fragments (Yang *et al.*, 1998). A negative control was included in the bone digestion and DNA extraction steps. The bone powder was suspended in 1 ml of digestion buffer (0.5 M EDTA pH 8.0, 1 M Urea and 100 µg/ml of proteinase K) and incubated overnight at 38°C with constant agitation. The samples were centrifuged for 5 minutes at 2000 rpm, and concentrated to 100 µl with Amicon columns (Millipore-Amicon Ultra-4 30 k Da, Merck, Germany) by centrifuging for 10-15 minutes at 4000 g. Each sample was then mixed with 5 volumes of PB buffer included in the commercial MinElute PCR Purification Kit from Qiagen (Qiagen Nordic, Sweden). Ancient DNA was recovered, following the manufacturer's protocol, in a final volume of 100 µl.

GENOMIC LIBRARY PREPARATION AND SEQUENCING

Double stranded libraries were prepared from 20 µl of DNA extract using the blunt-end ligation protocol described in (Meyer and Kircher, 2010) with modifications as in (Günther *et al.*, 2015).

The libraries were tested with real-time quantitative PCR (qPCR) to ensure equal representation of

indexed libraries while also confirming if the libraries were optimally diluted. This step provided the optimal number of PCR cycles (16 for chi004, 15 for chi008 and 11 for chi009) to be performed for each library. The index PCRs were set up in a total volume of 25 µL with a final concentration of 1X Gold Buffer (Invitrogen/Life technologies), 2.5 mM magnesium chloride, 0.25 mM dNTPs, 0.2 µM IS4 PCR primer (5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGTACACTCTT T C C C T A C A C G A C G C T C T T 3'), 0.2 µM indexing primer (5'-CAAGCAGAAGACGGCATAACGAGATxxxxxxxGTGACTGGAGTTCAGACGTGT 3', where x is one of the 228 different 7 bp indexes described in Meyer and Kircher, 2010), 0.1 U/µl of AmpliTaq Gold (Invitrogen/Life technologies) and 3 µl of DNA library. The cycling conditions were: 12 min activation step at 94°C, then the optimal number of cycles (16, 15 or 11) of 30 s at 94°C, 30 s at 60°C and 45 s at 72°C, a final extension step at 72°C during 10 min. For each DNA library we performed 4 PCR reactions and a blank, with a single index in the P7 primer. AMPure Beads (Beckman Coulter) were used to purify PCR products.

The purified libraries were quantified using an Agilent Bioanalyzer 2100 and a Qubit Fluorometer (Günther *et al.*, 2015). The Illumina HiSeq X Ten instrument was used for paired-end (2x100bp) shotgun resequencing, through service acquisition to the National Genomics Infrastructure (NGI, SciLifeLab) in Stockholm, Sweden.

DATA ANALYSIS

SEQUENCE DATA PRE-PROCESSING AND ANCIENT DNA AUTHENTICATION

Quality control and pre-processing

To inspect the quality of raw reads, we used the software Fastqc v.0.11.7. Fastqc provides a quality-control of

the reads generated by the sequencing platform. It produces a graphical report with summary statistics for the data set, such as per base sequence quality and over represented sequences (amongst others), giving a perspective on possible problems that the data may have, which we should be aware before subsequent analyses. Following, we used the AdapterRemoval software (version 2), that trims adapters of sequenced reads and does the subsequent merging of paired-end reads with an overlap of 11 bp. Reads shorter than 30 bp length and with a quality score less than 20 were discarded.

Preparation of the reference sequence, alignment, and damage patterns

We aligned the merged and trimmed reads to the reference sequences of interest: the reference mitogenome (NIH GenBank accession number: NC6853) and the *Bos taurus* reference genome version Btau_4.6.1 that contains an assembled Y-chromosome (bosTau7; GenBank accession number: GCA_000003205.4) for the biological sex determination.

We used the program BWA, specifically the BWA aln algorithm v.0.7.17-r1194. First, we indexed the reference sequence using the command *bwa index*, which is essential to querying the DNA reads to the reference. After, a reference dictionary was created. It contains information about the sequences, such as sequence length and name. To create the dictionary, we used Picard version. 2.18.5. For subsequent analyses, the reference sequence must be indexed with the command *samtools faidx* (SamTools version 1.8), as it facilitates access to the different regions within the reference genome.

For the alignment, we used the command *bwa aln*, giving as input the sample and the reference. We changed some parameters adequate for degraded samples, such as deactivating the seed-length (-l 1000), tolerating more gaps (-o 2) and allowing for more substitutions (-n 0.01), due to the damaged nature of ancient DNA molecules. In order to distinguish among samples, libraries and other technical information down the pipeline, we assigned predefined tags to specific sets of reads (Read Groups) using Picard. We marked and removed duplicates (REMOVE_DUPLICATES=True) with Picard MarkDuplicates v.2.18.5 (<https://broadinstitute.github.io/picard>), as it is common that they occur during the PCR amplification. Finally, we locally realigned regions where insertions and deletions may have caused misalignments, using GATK v.3.7.

To obtain a summary report with useful informa-

tion about our alignment, we used Qualimap version 2.2.1. It examines the alignment data in our bam files and provides an overview to detect bias in the sequencing or mapping processes.

The MapDamage software (v. 2.0) was then used to verify if the samples analysed show the typical ancient DNA deamination patterns. It computes nucleotide misincorporations and fragmentation patterns following the mapping of the reads to the reference genome.

PHYLOGENETIC ANALYSIS OF CATTLE MITOGENOMES

To obtain the consensus mitogenome sequences we used *doFasta* in ANGSD v.0.931-, to select the most frequent base per position with at least 3X depth of coverage and considering only reads with minimum quality of 20 and minimum mapping quality of 30. The positions which did not have enough coverage were classified as undetermined (and represented by "N").

The sequences were imported to Geneious Prime v.2021.1.1 with the Muscle alignment tool v. 3.8.425, for alignment with extant cattle mitochondrial sequences available in Genbank using the Muscle alignment tool v. 3.8.425. These sequences represent the major haplogroups of cattle matriline so we could assign our samples to these clades. The different haplogroups represented in our alignment were the taurine: T1, T2, T3, T4, T5, and Q; the zebu I; and the more ancestral, R and P lineages. The phylogenetic analysis was done with RAXML (Appendix I - Table S1) v. 8.1.7 and the GTR+GAMMA evolutionary model.

SEX DETERMINATION

For the biological sex determination of the Chibanes specimens, we used the method described in (Skoglund *et al.*, 2013). The method allows to determine the biological sex and classify samples as "female" or "male" with 95% confidence intervals. The classifications depend on what is denoted as R_y , calculated as a fraction of the number of reads mapped to the Y chromosome (n_y) and the total number of reads mapped to both sex chromosomes ($n_x + n_y$).

Results

WHOLE-GENOME RESEQUENCING RESULTS

The three samples analysed contained high endogenous DNA content ranging from 1.03 to 25.86% (Table 2). It is important that we obtained these percentages since samples with over 1% endogenous

Table 2 - Endogenous DNA content and summary statistics of the mitogenome resequencing results obtained for Chibanes' specimens.

Sample	Endogenous DNA (%)	Mitochondrial DNA reads*	Mean coverage mitogenome (X)	Mitogenome coverage (>3X) (%)
Chi004	25.86	249870	465.81	100
Chi008	2.48	10794	18.65	99.71
Chi009	1.03	2818	9.86	99.26

*mapping quality > 25

Table 3 - Biological sex determination results.

Sample	Biological sex	Ry value	Mapped X-chromosome reads	Mapped Y-chromosome reads	Confidence Interval (CI)
chi004	Female	0.0003	549823	153	0.0002-0.0003
chi008	Male	0.0369	38130	1462	0.0351-0.0388
chi009	Female	~ 0	26691	1	0.0-0.0001

DNA are generally selected for deeper resequencing which allows to increase the coverage and improve the quality of subsequent population genomic analyses particularly for nuclear DNA.

VALIDATION OF ANCIENT DNA SEQUENCES

To authenticate our results, we needed to assess if our samples were indeed ancient, as suggested by the archaeological context. As mentioned before, we used the MapDamage software to assess degradation patterns.

The map damage results obtained for the Chibanes' samples (Figure 3) corresponded to the expected, i.e. ancient DNA profiles are characterized by an increased frequency of cytosine to thymine and complementary guanine to adenine misincorporations at the 5' and 3' -ends, respectively, caused by deamination (Dabney *et al.*, 2013). Also, DNA fragmentation was high (read lengths < 100 bp; Appendix II - Figures S1 and S2), probably due to hydrolytic depurination. Thus, we can say with certainty that these are in fact ancient samples, and not contaminant DNA from more recent bones that could have been buried at the

historical site or from the reagents used during the DNA extraction and genomic library preparation.

MITOGENOME ANALYSIS OF CATTLE FROM CHIBANES

In cattle, mitochondrial DNA is geographically structured, which makes it possible to associate maternal lineages to specific geographic regions (McHugo *et al.*, 2019). In the phylogenetic tree below, reference mitogenomes representative of the major haplogroups (including Portuguese native cattle) and Chibanes' samples (chi004, chi008 and chi009) are depicted (Figure 4).

Phylogenetic analysis showed that samples chi004 and chi009 belong to the African-T1 haplogroup and sample chi008 belongs to the European-T3 haplogroup. Since the T1 haplogroup was detected in the Iberian Peninsula, in earlier times, such as the Neolithic (Colominas *et al.*, 2015) and in more recent times (17th century) (Ureña *et al.*, 2021), the presence of the African matriline in Chibanes suggests that there was genetic continuity of maternal lineages through time between extant and ancient cattle

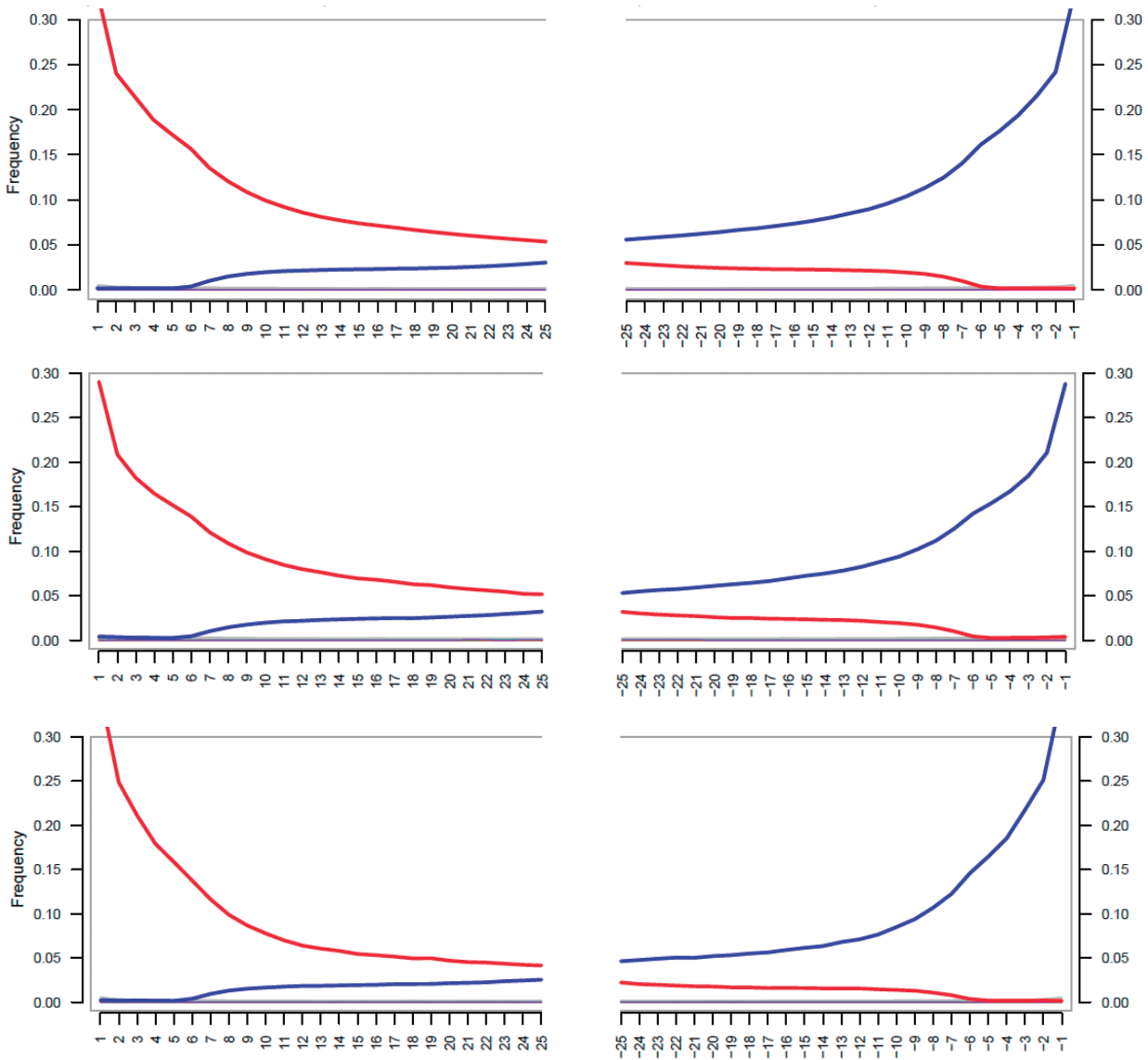


Figure 3 - Graphs showing typical ancient DNA deamination patterns for samples chi004, chi008 and chi009, i.e. an excess of cytosine to thymine (C to T) misincorporations at 5' ends (left), and complementary guanine to adenine (G to A) misincorporations at 3' ends (right). (red-thymine, blue-adenine).

in the Iberian Peninsula. Such continuity of maternal lineages associated with a founder bottleneck is consistent with what has been shown for cattle from other regions (Lenstra *et al.*, 2014).

Whole-genome nuclear data will be an important tool in the future to better understand this consistent African influence in the Iberian Peninsula, infer about the role of paternal lines in gene flow and the genetic affinities between the different Mediterranean cattle breeds.

WHAT WAS THE SEX OF THESE CHIBANES CATTLE?

As for the biological sex determined for each sample, the results revealed chi004 and chi009 to be

females, as they were assigned with high confidence (95% confidence interval (CI), $R_y < 0.0003$; Table 3). As for chi008, the value of $R_y = 0.0369$ suggests it was a male as the confidence interval did not overlap the assignment threshold of females.

Conclusions and future work

The expansion of the Roman Empire had a big impact on the culture and economy of Lusitania, despite its peripheral location. There has been an intense debate about the role played by animal husbandry and livestock trade during the different phases of the Roman Period. For example, there is zooarchaeological evidence showing the Romans improved their cattle for larger body sizes in newly

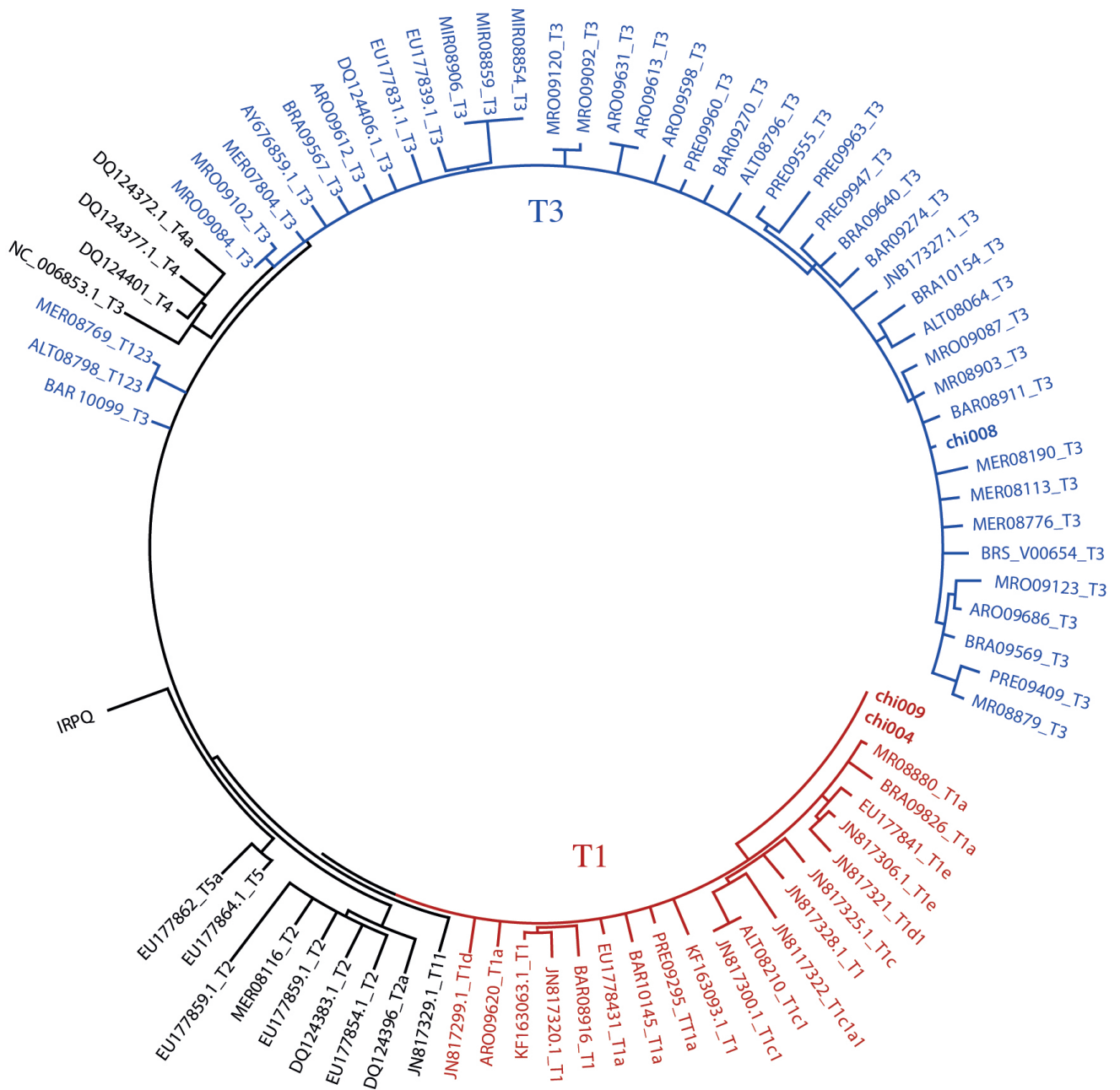


Figure 4 - Phylogenetic tree of cattle mitogenomes obtained with RAxML. The T1 and T3 haplogroups are shown in red and blue, respectively, while other maternal lineages are in black.

founded cities of the province of Lusitania (such as *Emmerita Augusta* and *Ammaia*) (Detry *et al.*, 2021). However, in some early settlements, dated to the Roman Republican (2nd/1st centuries BC), a substantial increase in the size of cattle was not observed, suggesting that local resources were not improved. Still, some sites, such as Chibanes and Mesas do Castelhinho (see Figures 1 and 2), did show slightly higher averages of widths and lengths in cattle bones, than contemporaneous settlements such as Alcáçova de Santarém. This study is focused on

Castro de Chibanes, in Palmela, a fortification occupied by the Romans in the time span of c. 125-40 BC. We generated whole genome shotgun resequencing data (Illumina HiSeqX) for cattle remains collected in Chibanes (one metatarsal and two inferior third molars). Endogenous DNA content of these specimens varied between 1.03% and 25.86% and the percentage of the mitogenome covered at a depth above 3X was greater than 99.26%. Archaeogenomic and zooarchaeological data suggest that the Romans may have improved local cattle from Chibanes.

Further whole-genome analyses are needed to determine if new stock was also used.

The present study shows that cattle from Chibanes were mostly of the African-T1 type (despite the low sample size considered here), which supports previous studies that state that North African taurine cattle had a significant influence in cattle from the Iberian Peninsula (in this case specifically in Portugal) (Da Fonseca *et al.*, 2019). We did not detect new matrilineages, thus it appears that new lineages of cattle were not introduced in Chibanes by the Roman troops. Combining these findings with zooarchaeological data suggests that cattle were instead locally improved by the Romans. As for future work, whole-genome analyses need to be carried out to determine if the Romans introduced new stock (e.g. male mediated gene flow) and also whether they selected specific traits to improve local cattle, such as coat colour, genes linked to body size, meat and milk production traits.

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Appendices

Appendix I. Mitogenome reference sequences used in the phylogenetic tree.

Tabela S1 - Mitogenome references, corresponding haplogroups and reference.

Assession number	Haplogroup	Reference
AY676859.1	T3	Shahid (2004)
GU256940	I	Shahid (2004)
BRS_V00654	T3	Anderson (1982)
DQ124372	T4a	Shin (2005)
DQ124377.1	T4	Shin (2005)
DQ124383.1	T2	Shin (2005)
DQ124389	P2	Shin (2005)

DQ124396	T2a	Shin (2005)
DQ124401	T4	Shin (2005)
DQ124406.1	T3	Shin (2005)
EU177831.1	T3	Achilli (2008)
EU177839.1	T3	Achilli (2008)
EU177841	T1	Achilli (2008)
EU177854.1	T2	Achilli (2008)
EU177858.1	T2	Achilli (2008)
EU177859.1	T2	Achilli (2008)
EU177862	T5	Achilli (2008)
EU177864.1	T5	Achilli (2008)
EU177867.1	Q	Achilli (2008)
EU177868	I1	Achilli (2008)
EU177870	I2	Achilli (2008)
FJ971081	Q	Achilli (2009)
FJ971084	R	Achilli (2009)
FJ971085.1	R	Achilli (2009)
FJ971087	R	Achilli (2009)
GU985279	P1	Edwards (2010)
HQ184031	Q2	Bonfiglio (2010)
HQ184034	Q1a	Bonfiglio (2010)
HQ184039	Q1	Bonfiglio (2010)
JN817320	T1b1	Bonfiglio (2012)
JN817321	T1d1	Bonfiglio (2012)
JN817322	T1c1a1	Bonfiglio (2012)
JN817325.1	T1c	Bonfiglio (2012)
JN817327.1	T1b	Bonfiglio (2012)
JN817328.1	T1c	Bonfiglio (2012)
JQ437479	P1	Lipinski (2012)
KF163063.1	T2	Horsburgh (2013)
KF163093.1	T1	Horsburgh (2013)
KF525852	C	Zhang (2013)
NC_006853.1	T3	Chung (2005)
ALT08064	T3	Fonseca (2019)
ALT08207	Q	Fonseca (2019)
ALT08210	T1c1	Fonseca (2019)
ALT08796	T3	Fonseca (2019)
ALT08798	T123	Fonseca (2019)
ALT09280	T1c1	Fonseca (2019)
ARO09598	T3	Fonseca (2019)
ARO09612	T3	Fonseca (2019)
ARO09613	T3	Fonseca (2019)
ARO09620	T1a	Fonseca (2019)
ARO09631	T3	Fonseca (2019)
ARO09686	T3	Fonseca (2019)
BAR08911	T3	Fonseca (2019)
BAR08916	T1	Fonseca (2019)
BAR09270	T3	Fonseca (2019)
BAR09274	T3	Fonseca (2019)
BAR10099	T3	Fonseca (2019)

BAR10145	T1a	Fonseca (2019)
BRA09567	T3	Fonseca (2019)
BRA09569	T3	Fonseca (2019)
BRA09640	T3	Fonseca (2019)
BRA09826	T1a	Fonseca (2019)
BRA10154	T3	Fonseca (2019)
MRO09084	T3	Fonseca (2019)
MRO09087	T3	Fonseca (2019)
MRO09092	T3	Fonseca (2019)
MRO09102	T3	Fonseca (2019)
MRO09120	T3	Fonseca (2019)
MRO09123	T3	Fonseca (2019)
MER07804	T3	Fonseca (2019)
MER08113	T3	Fonseca (2019)
MER08116	T2	Fonseca (2019)

MER08190	T3	Fonseca (2019)
MER08769	T123	Fonseca (2019)
MER08776	T3	Fonseca (2019)
MIR08854	T3	Fonseca (2019)
MIR08859	T3	Fonseca (2019)
MIR08879	T3	Fonseca (2019)
MIR08880	T1a	Fonseca (2019)
MIR08903	T3	Fonseca (2019)
MIR08906	T3	Fonseca (2019)
PRE09295	T1a	Fonseca (2019)
PRE09409	T3	Fonseca (2019)
PRE09555	T3	Fonseca (2019)
PRE09947	T3	Fonseca (2019)
PRE09960	T3	Fonseca (2019)
PRE09963	T3	Fonseca (2019)

Appendix II. Single end read length distribution plots.

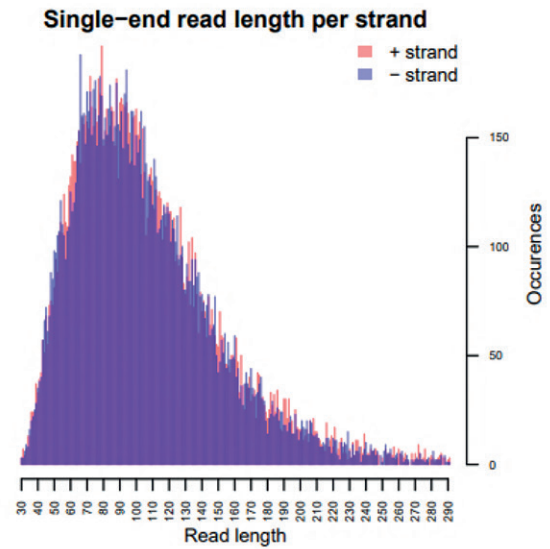
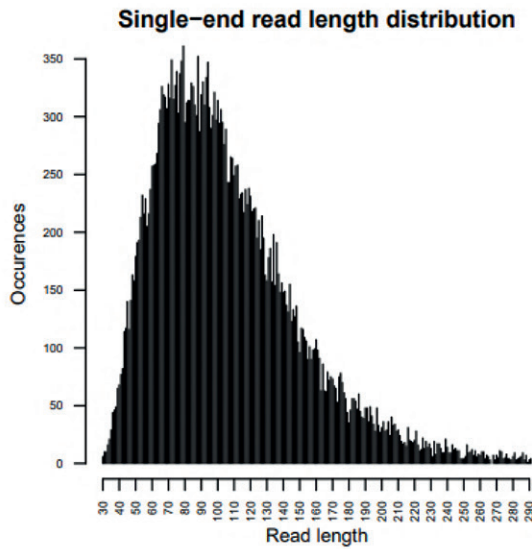


Figure S1 - Read length distribution plot - sample chi004.

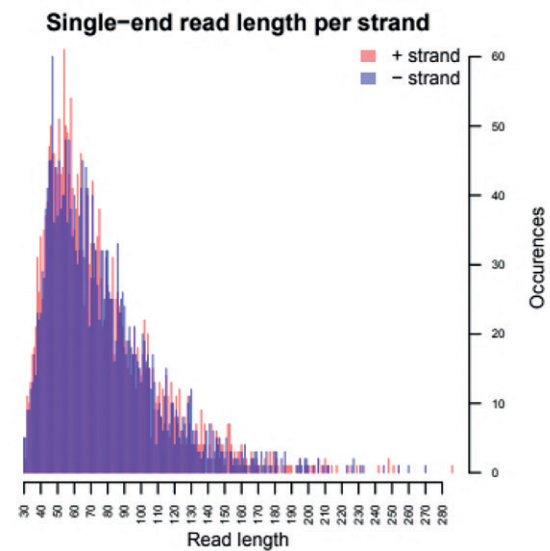
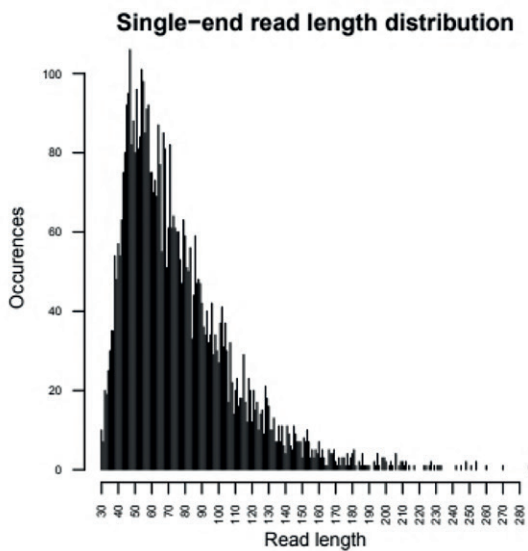


Figure S2 - Read length distribution plot - sample chi008.

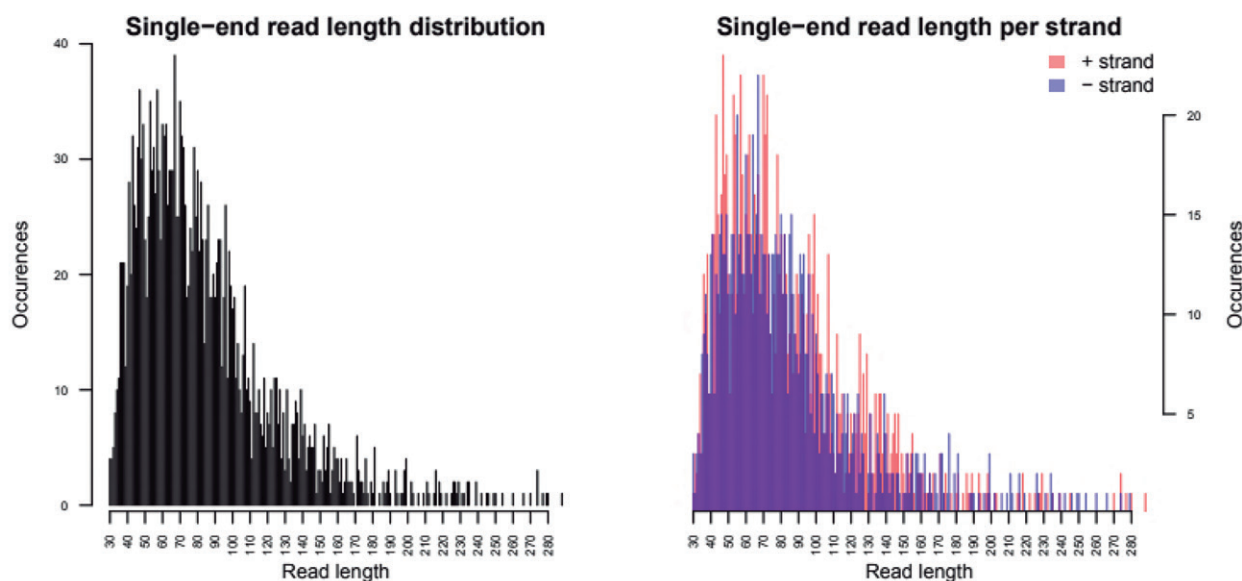


Figure S3 - Read length distribution plot - sample chi009.

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