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Roman dogs from the Iberian Peninsula and the Maghreb – A glimpse into their morphology and genetics

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ABSTRACT

In this study, we integrate osteometric and palaeogenetic data to investigate dog variability in the Roman Empire in Iberia and North Africa. Osteometry was used to distinguish the status—domestic or wild, of approximately 2000 years old *Canis* remains and to understand to what extent teeth and long bones varied in dogs in the Roman provinces of *Mauretania Tingitana*, *Lusitania* and *Tarraconensis*. High-throughput 454-DNA sequencing technology was used to obtain mitochondrial DNA (mtDNA) sequences from 15 bone and teeth samples. We identified five dog haplotypes from partial sequences of the hypervariable *D-loop* region. MtDNA haplotypes were grouped into two of the four major clades found in present-day dogs. We detected three clade A haplotypes in 12 samples from Portugal, Spain and Morocco, and a single clade D haplotype in 3 samples from Spain. So far, this is the oldest evidence for the presence of dog clade D in Iberia. It is dated to the late Roman occupation in the 4th–5th cent. AD (ca. 1,600 years ago).

Our results confirm the existence of distinct dog morphotypes in Roman times that also harboured distinct genetic lineages. According to our data, dogs from distinct mtDNA lineages (clades A and D) have been continuously bred in the Iberian Peninsula since at least 1600 years ago. Moreover, the sharing of matrilineal lineages between dogs from Spain and North Africa may indicate gene flow. Dogs could have been easily transported between these regions by humans along maritime and terrestrial trade routes. These results provide new insights into pre-Roman and Roman domestication practices, confirming selection practices were extensively applied to dogs during the first centuries of our era in the Iberian Peninsula. We show that the greater size variability of teeth length (and consequently cranium) and long bone breadths (and consequently phenotype) of Roman dogs in the Iberian Peninsula, is concomitant with the detection of diverse and rare maternal lineages. This would reflect an intensification of dog breeding and the use of non-local dogs for breeding.

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1. Introduction

Dogs (*Canis lupus familiaris*), the oldest domesticated, show huge morphological variability today. But when did this domestic animal begin to vary so much in size and shape? A variety of dog statures, with small dogs having a withers height less than 30 cm and others

over 60 cm, have been reported from the Late Glacial Period ca. 18,000–10,000 years ago (Horard-Herbin et al., 2014). Although not as variable as extant dogs, greater variability in both size and shape is also well known in dogs from Roman times ca. 2,000 years ago (Altuna and Mariezkurrena, 1992; Bennett et al., 2016; Colominas, 2015; De Grossi Mazzorin and Tagliacozzo, 2000; MacKinnon, 2010; Peters, 1998). This process probably originated in preceding times, i.e., in the late Iron Age ca. 2,500 years ago (Bennett et al., 2016), but the very small lap, or toy dogs, appear for the first time, and in large numbers, throughout the Roman Empire ca. 2,000 years ago (e.g. Boessneck and von den Driesch, 1980; Altuna and Mariezkurrena, 1992; Fernández-Rodríguez, 2003; MacKinnon, 2010). In the Western Mediterranean, the differentiation of dog populations into different phenotypes similar to breeds may have its roots in the Roman period (De Grossi Mazzorin and Tagliacozzo, 2000, 1998). In fact, the extent of morphological variation is most evident in Roman times and today—perhaps because artificial selection was and is extensively exercised over dogs by humans for aesthetic/emotional reasons (e.g. dogs just for companionship such as lap dogs) and not only for functional/working purposes (dogs used for hunting or herding). The definition of today's dog breeds follows criteria defined in the 19th century AD and it is not possible to recognize extant dog breeds in ancient periods merely through osteological morphology (Harcourt, 1974; Morales et al., 2015). Nevertheless, Varro in his *De re rustica* book (1st cent. AD) provided a dog classification based on their function.

Coat colour also varied since early times. The investigation of molecular markers associated with dark versus light coat colour, i.e., *Mc1r* (melanocortin 1 receptor) and *CBD103* (canine- β -defensin) genes, showed the presence of dogs with light and dark coat colours as early as the beginning of the Mesolithic, over 8000 years ago (Ollivier et al., 2013). Well known Roman iconography—mosaic floors and wall paintings—confirm a diversity of morphologies and coat colours in dogs (Fig. 1). The dog morphotypes represented in mosaics do not necessarily reflect local dogs, as the artisans tended to move around the Empire possibly producing representations of what they had seen in other places; however, in the Roman city of Conímbriga, in Lusitania, Portugal, there are indications that the artisans were local (Correia, 2013). Thus, it is probable that these dog morphotypes existed across the Roman Empire.

Zooarchaeological studies aid our understanding of size and morphological variation in Roman *Canis* (e.g., Altuna and Mariezkurrena, 1992; Bennett et al., 2016; Colominas, 2015; MacKinnon, 2010). The various anatomical parts are preserved differently in the archaeological record. For instance, the first molar tooth— M_1 —is frequently found intact in the archaeological record. However, the M_1 length variability is not necessarily correlated with body size, but it reflects to some extent the cranium size and shortening of the snout. This latter characteristic is a marker of the domestication process, since dogs tend to have shorter snouts than wolves (Tchernov and Valla, 1997). Measurements of the long bones (femura) are a better proxy for the overall body size variability (Wayne, 2001) and therefore can indicate the presence of different morphotypes. However, in the archaeological record these bones are frequently found fragmented.

Genetic studies using ancient DNA (aDNA) can provide important insights into the understanding of population genetics, gene flow and human-driven selection for certain traits in animals of the past (Botigué et al., 2017; Ollivier et al., 2016, 2013; Pilot et al., 2014; Svensson et al., 2007), particularly when bones are morphologically indistinct. However, these kinds of studies are still scarce for Roman material, particularly in the Iberian Peninsula, a region where samples tend to be poorly preserved (but see Catagnano, 2016). Mitochondrial DNA (mtDNA) has been particularly useful for studying the genetic composition of archaeological specimens (e.g.,

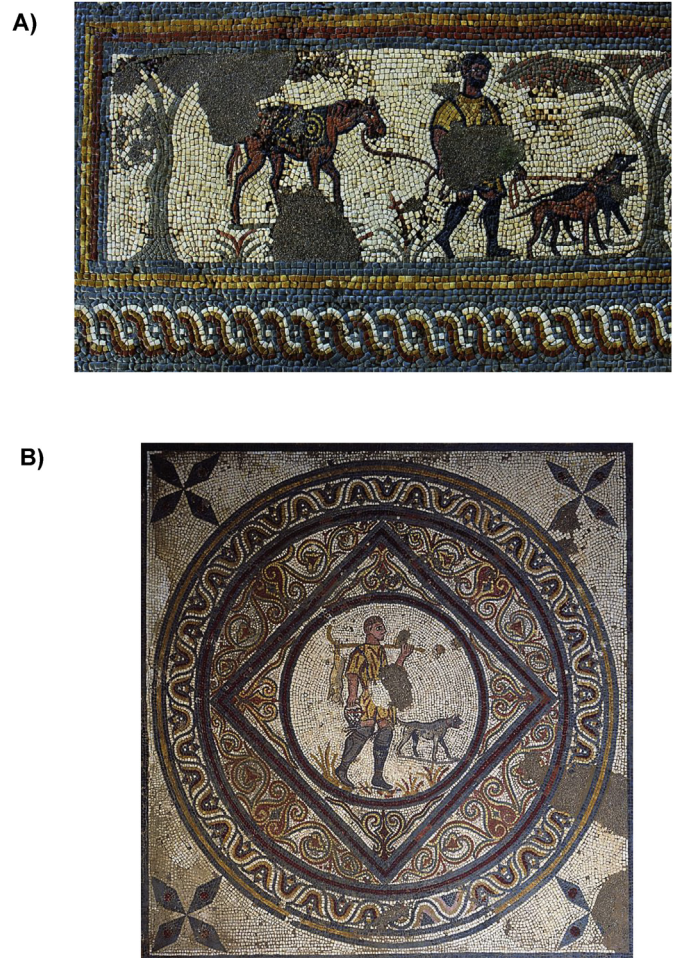


Fig. 1. Details of Roman mosaics from Conímbriga (Portugal) depicting dogs with different shape and colour: **A)** Two dogs on a leash with different coat colours, one brown and the other black (detail of mosaic n° 11 of Casa dos Repuxos, (Oleiro, 1992), Photo by Humberto Rendeiro©MMC/DGPC); **B)** A hunter carrying a hare and accompanied by a dog with a short snout (detail of mosaic n° 15 of Casa dos Repuxos, (Oleiro, 1992), Photo by Delfim Ferreira©MMC/DGPC). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Frantz et al., 2016; Leonard et al., 2002), especially in hot climates where DNA is poorly preserved. MtDNA sequence variability does not reflect directly variation in morphological traits, but regions such as the D-loop generally show a large degree of sequence variation between individuals, and it is most useful for addressing intraspecific evolutionary questions (e.g. Frantz et al., 2016; Leonard et al., 2002).

Worldwide mtDNA diversity of extant dogs indicate the existence of six clades or haplogroups: A, B, C, D, E and F (Savolainen et al., 2002). Clades A, B, C and D represent the majority of the maternal diversity found in dogs. Clade A is the most frequent lineage currently observed in dogs from Europe (including Iberia) and North Africa, whereas clade D is the less represented and geographically restricted to Portugal, Spain, Morocco, Turkey and Scandinavia in present times (Ardalan et al., 2011; Pires et al., 2006; Savolainen et al., 2002). Extant dogs from Iberia and North Africa have high genetic diversity, carrying haplotypes belonging to clades A, B, C and D (Pires et al., 2006). Clades E and F were only detected in dogs from Asia. Concerning clade D in ancient European dogs, it was first detected in the Near East and later in South-eastern and Central Europe (Pionnier-Capitan, 2010) during the Neolithic ca. 10,000 BC. This clade would most likely have been introduced in

Europe in the Neolithic along with other domesticated animals used for food, such as pig, sheep, goat and cattle (M. Ollivier, pers. com.).

In this study, we integrate osteometric, morphological and palaeogenetic data to characterize dog phenotypic and genetic variability of dogs of Roman times from Iberia and North Africa. Our hypothesis is that the greater morphological variation in dogs attributed to the Romans coincides with the presence of various dog mtDNA lineages. This would be in line with the Romans' interest in improving their domesticated animals—known to be more efficient and diverse, even extravagant.

2. Material and methods

2.1. Zooarchaeological study

The sites considered cover a time span from the Mesolithic (*ca.* 7,500 years ago) to present day dogs and wolves in order to assess the diversity of dog sizes in each period including Roman, and how it compares with extant populations. We included measurements of the lower first molar tooth (M_1) length and the humerus distal breadth of dog specimens from 56 archaeological sites including 13 sites dated to the Roman period (Tables 1 and 2). We selected the M_1 and distal humerus because these parts are frequently found in archaeological sites. M_1 crown length and humerus distal breadth were measured following von den Driesch (1976).

Lengths of archaeological specimens of M_1 were compared with measurements taken from Portuguese reference collections of skeletons of extant wolves ($n = 45$, 19 males, 18 females and 8 of unknown sex) and dogs ($n = 29$, unknown sex). These Portuguese collections are held at Laboratório de Arqueociências, Direcção Geral do Património Cultural, Museu Nacional de História Natural e da Ciência and the Laboratório de Anatomia, Universidade Lusófona de Humanidades e Tecnologias, in Lisbon. In dogs, sexual dimorphism is present but it is not very strong (Cruz, 2007), and therefore this parameter does not affect size greatly. Adding to that, the variation of phenotypes and breeds also overlap any size variation derived from sexual dimorphism. Consequently, we did not consider sexual dimorphism as a variable in the analysis.

We measured the tooth and humerus from reference collections in Portugal and Spain and archaeological collections from Portugal. Measurements taken by other authors were used for comparisons (see Tables 1 and 2). The Coefficient of Variation (CV), being $CV = (\text{standard deviation/average}) * 100$, for the M_1 length and humerus breadth was calculated for each period.

We used the non-parametric test of Siegel-Tukey (Siegel and Tukey, 1960) in order to test whether differences between the populations existed regarding the diversity of the measurements. This test measures the dispersion of values in two populations, that is, whether a population is more homogeneous than another.

3. Genetic study

3.1. Archaeological sites

The dog remains of this study derive from three archaeological sites located in the western range of the Roman Empire, namely (from south to north): Thamusida in northern Morocco (*Mauretania Tingitana*) in 1999; Monte Molião, in Lagos in southern Portugal (*Lusitania*) in 2006/2008; and Lugo in Galicia in northern Spain (*Tarraconensis*) in 2003. The exact location of these sites and the sampled materials are shown in Fig. 2. A description of the archaeological sites from where these samples were excavated is presented below. It should be noted that the archaeological remains of dog from Morocco and Spain are contemporaneous (3rd-

4th cent. AD - *ca.*1,600 years ago) whereas those from Portugal are older (1st cent. BC-1st cent. AD - *ca.* 2,000 years ago).

Thamusida (Morocco) The ruins of the city, already known from the end of the 19th century AD, were systematically investigated in the course of the 1930s and 1960s. Archaeologists discovered the most ancient layers of Mauro settlement and the main Roman monuments. In the year 40 AD, the province of *Mauretania Tingitana* was founded by Rome; the ancient Mauro settlement was destroyed during military operations aimed at controlling the region. During the second half of the 1st century AD, a military camp, close to a ford on the Sebou River, was established there. Around this camp a new urban centre was born. This new small city included different facilities such as houses, baths, temples, taverns and shops. Walls were constructed during the second half of the 2nd cent. AD. These were probably needed to resist attacks from the Berber tribes of the Gharb (west). It is also worth noting that at the end of the 3rd century AD the Roman army left the area, but the site continued to be inhabited. Later on, during the 7th-8th cent, Arabs arrived at this settlement, but they chose to live outside the ancient city walls.

Monte Molião (Lagos, Portugal) was a small Roman town located on the south coast of Portugal, which was built over an Iron Age *oppidum*. The Roman occupation began in the 2nd century BC (Republican period), with intensive connections to other Mediterranean areas including the Italian Peninsula, North Africa and Bay of Cadiz, and lasted until the end of the 2nd century AD (Imperial period). Chronologies were obtained from the datable imported materials (ceramics) found on the site. Trading between Monte Molião and North Africa existed, namely with the area of present-day Tunisia during the Republican and the Imperial Roman periods, as attested through imported food products transported in amphorae (Arruda and Sousa, 2013) as well as the presence of abundant imported ceramics from this area (Arruda, 2007). Dog remains were found in layers related to the site's occupation, especially the Imperial period (1st -2nd century A.D.; Detry and Arruda, 2013).

Lugo (Lucus Augusti) (Spain) was a Roman city that developed during the Early Imperial period (late 1st century BC) in the north-western region of the Iberian Peninsula. It eventually became *Conventus Lucensis* capital, one of the administrative units in which the *Gallaecia* province was divided (Rodríguez Colmenero, 2011). Excavations at the site *Domus del Mitreo*, revealed a diachronic occupation since the Roman period. Regarding the oldest records, they reflect a construction of the Early Imperial period, and a *domus* identified as a *mitreo* (for cult) was recorded (Alvar et al., 2006; Rodríguez and Cordeiro, 2014; Rodríguez Cao, 2006). This construction was renewed several times and it was damaged due to a Roman wall construction in the late 3rd cent. AD. It eventually disappeared at the end of 3rd cent. - 4th cent. AD during a refurbishment of the city. Dog remains excavated from deposition levels related to the renovation and destruction of the *domus*, can be dated to the 4th century, as indicated by other associated archaeological materials. The presence of camelids (possibly dromedary) among the faunal remains at *Domus del Mitreo* excavation site (C Fernandez-Rodriguez, pers. com.) may reflect connections with North Africa. There are also references to other parts of the Lugo city for the presence of North African products (Naveiro López, 1991) such as amphorae (Carreras Monfort, 2011; Naveiro López, 1991).

3.2. Sub-sampling for aDNA analysis

A total of 15 dog remains were sampled for ancient DNA (aDNA) analyses: one upper third pre-molar from Thamusida; two proximal femora and a mandible fragment from Monte Molião; and two

Table 1
Measurements of the lower first molar tooth (M_1) from various archaeological sites in Portugal, Spain, Morocco and Tunisia. N – number of specimens measured; Min. – minimum value; Max. – maximum value; SD – Standard Deviation; CV – Coefficient of Variation (%).

Archaeological Site	Location	Country	Roman province	Chronology	N	Min.	Max.	Average	SD	CV (%)	References
20th Century Wolves		Portugal			45	23.8	30.7	27.3	1.5	5.7	(Detry and Cardoso, 2010)
20th Century Dogs		Portugal and Spain			29	14.6	25.3	19.4	3.0	15.5	C. Detry, personal communication
Silos de Carnide	Lisboa	Portugal		Modern Period	8	17.9	20.2	18.4	1.0	5.3	C. Detry and S.J.M. Davis, personal communication
Mosteiro de Santo André	Amares	Portugal		Modern Period	1	17.9	17.9	0	0.0	0.0	Davis, unpublished
Mosteiro do Bouro	Amares	Portugal		Modern Period	2	19.7	22.1	20.9	1.2	5.7	Davis, unpublished
Lixeira	Silves	Portugal		Modern Period	11	17.9	22.1	18.8	1.4	7.4	
Alcáçova de Santarém	Santarém	Portugal		Medieval Islamic	3	22.6	23.1	22.8	0.2	1.0	(Davis et al., 2008)
		Portugal		Medieval Islamic	1	23.8	23.8	0.0	0.0	0.0	(Davis, 2006)
				Medieval Period	4	22.6	23.8	23.0	0.5	2.2	
Astorga	Astorga	Spain	Tarraconensis	Late Roman (3rd–4th cent. AD)	14	16.1	23.7	20.5	2.5	12.2	(Fernandez-Rodriguez, 2000)
Els Mallols	Barcelona	Spain	Tarraconensis	Late Roman (3rd–4th cent. AD)	3	19.5	21.0	20.2	0.6	3.1	(Catagnano, 2016)
Odrinhas	Sintra	Portugal	Lusitania	Late Roman (3rd–4th cent. AD)	14	15.6	22.0	19.0	2.0	10.5	(Davis and Gonçalves, 2017)
Monte Molião	Algarve	Portugal	Lusitania	Imperial Roman (1st–2nd cent. AD)	1	17.5	17.5	0.0	0.0	0.0	(Detry and Arruda, 2013)
Thamusida		Morocco	Mauretania Tingitania	Imperial Roman (1st–2nd cent. AD)	3	19.2	19.9	19.6	0.3	1.5	(De Grossi Mazzorin and De Venuto, 2010)
Calle Almendralejo	Mérida	Spain	Lusitania	Imperial Roman (1st–2nd cent. AD)	50	14.8	22.7	19.2	1.8	9.6	C. Detry and F.J. Heras, personal communication
Alcáçova de Santarém	Santarém	Portugal	Lusitania	Republican Roman (1st–2nd cent. BC)	1	20.6	20.6	0.0	0.0	0.0	(Davis, 2006)
Odemira	Odemira	Portugal	Lusitania	Republican Roman (1st–2nd cent. BC)	1	26.0	26.0	0.0	0.0	0.0	(Davis and Vilhena, 2017)
				Roman Period	87	14.8	26	19.5	2.1	10.8	
Althiburos		Tunisia		Iron Age	3	18.4	21.7	20.5	1.5	7.3	(Valenzuela-Lamas, 2016)
R. dos Correeiros	Lisbon	Portugal		Iron Age	1	19.0	19.0	0.0	0.0	0.0	(Detry et al., 2016)
Hereuet	Tarragona	Spain		Iron Age	3	22.4	23.3	22.9	0.4	1.7	S. Valenzuela-Lamas, personal communication
Estinçells	Urgell	Spain		Iron Age	1	26.7	26.7	0.0	0.0	0.0	(Valenzuela-Lamas, 2010)
Alorda Park	Barcelona	Spain		Iron Age	2	22.8	23.0	22.9	0.0	0.0	(Valenzuela-Lamas, 2008)
Cerro Macareno	Sevilla	Spain		Iron Age	4	20.0	23.0	21.4	1.4	6.5	(Amberger, 1985)
Cerro de la Tortuga	Malaga	Spain		Iron Age	1	22.0	22.0	0.0	0.0	0.0	(von den Driesch, 1973)
Mas Castellar	Gerona	Spain		Iron Age	6	15.5	21.6	19.6	2.0	10.2	(Catagnano, 2016)
Los Toscanos	Malaga	Spain		Iron Age	1	19.0	19.0	0.0	0.0	0.0	(von den Driesch, 1973)
				Iron Age	22	15.5	26.7	21.2	2.3	10.8	
Castelon Alto	Granada	Spain		Bronze Age	4	18.0	21.0	19.9	1.1	5.7	(Milz, 1986)
Cerro de la Encina	Granada	Spain		Bronze Age	4	20.2	21.3	20.7	0.5	2.4	(Friesch, 1987)
Terrena del Reloj	Granada	Spain		Bronze Age	3	18.5	20.5	19.7	0.8	4.3	(Milz, 1986)
Fuente Álamo	Almeria	Spain		Bronze Age	3	19.5	21.5	20.3	0.8	4.2	(von den Driesch et al., 1985)
Purullena	Granada	Spain		Bronze Age	8	17.2	23.0	20.5	1.8	8.5	(Lauk, 1976)
Monachil	Granada	Spain		Bronze Age	2	20.5	21.7	21.1	0.6	2.8	(Lauk, 1976)
Cabezo Redondo	Alicante	Spain		Bronze Age	5	16.5	20.8	18.7	1.5	7.8	(Boessneck et al., 1969)
Sacaosjos Bei la Baneza	Léon	Spain		Bronze Age	3	22.5	23.5	23.0	0.4	1.8	(Boessneck and von den Driesch, 1980)
Los Palacios	Ciudad Real	Spain		Bronze Age	2	19.5	21.0	20.3	0.8	3.7	(Boessneck and von den Driesch, 1980)
Azuer	Ciudad Real	Spain		Bronze Age	5	17.5	21.5	20.4	1.5	7.2	(Boessneck and von den Driesch, 1980)
Lloma de Baxi		Spain		Bronze Age	2	19.7	23.0	21.4	1.7	7.7	(Sanchis and Sarrión, 2004)
				Bronze Age	41	16.5	23.5	20.4	1.6	7.8	
Leceia	Oeiras	Portugal		Chalcolithic	2	19.0	20.0	19.5	0.4	1.9	(Pires et al., 2001)
Zambujal	Torres Vedras	Portugal		Chalcolithic	5	17.7	23.0	20.6	0.0	0.0	(von den Driesch and Boessneck, 1976)
P. Torrão	Alentejo	Portugal		Chalcolithic	2	20.3	20.6	20.5	0.2	0.7	Davis, unpublished
Paraíso	Alentejo	Portugal		Chalcolithic	2	21.0	22.0	21.5	0.5	2.3	Davis, unpublished
Camino de las Yeseras	Madrid	Spain		Chalcolithic	12	18.1	22.2	20.2	1.4	6.7	(Daza, 2015)
Cerro de la Virgen	Granada	Spain		Chalcolithic	5	18.2	21.0	19.9	0.0	0.0	(von den Driesch, 1972)
Marizulo	Guipuzcoa	Spain		Chalcolithic	1	21.4	21.4	0.0	0.0	0.0	(Altuna, 1967)
Camino del Molino	Murcia	Spain		Chalcolithic	56	17.2	21.8	20.0	1.0	5.0	(Catagnano, 2016)
				Chalcolithic	83	17.2	23	20.1	1.1	5.5	
SJA Portam Latinam	Bilbao	Spain		Neolithic	1	18.0	18.0	0.0	0.0	0.0	(Altuna and Mariezkurrena, 2007)
Cova de L'Or	Alacant	Spain		Neolithic	1	20.0	20.0	0.0	0.0	0.0	(Sanchis and Sarrión, 2004)
Sierra del Mas Bonet	Gerona	Spain		Neolithic	1	21.0	21.0	0.0	0.0	0.0	(Catagnano, 2016)
Lameiras	Sintra	Portugal		Neolithic	1	20.7	20.7	0.0	0.0	0.0	S.J.M. Davis personal communication
Belas	Sintra	Portugal		Neolithic	1	20.0	20.0	0.0	0.0	0.0	Davis, unpublished
				Neolithic	5	18.0	21.0	19.9	1.0	5.0	

Table 1 (continued)

Archaeological Site	Location	Country	Roman province	Chronology	N	Min.	Max.	Average	SD	CV (%)	References
Muge (C. Arruda & C. Amoreira)	Salvaterra de Magos	Portugal		Mesolithic	2	20.5	21.6	21.1	0.6	2.6	(Detry and Cardoso, 2010)
Sado (Poças de São Bento)	Alcácer do Sal	Portugal		Mesolithic	1	21.7	21.7	0.0	0.0	0.0	C. Detry and S.J.M. Davis, personal communication
				Mesolithic	3	20.5	21.7	21.3	0.5	2.6	

tibiae, four humeri, two radii, one scapula, one pelvis and one femur from Lugo. These specimens were labelled and stored in individual zip-lock plastic bags and kept separately from modern samples.

We followed specific protocols for sub-sampling and aDNA extraction and took adequate precautions to avoid contamination: sub-sampling of archaeological remains was performed in the aDNA dedicated facilities at PALGENE, French National Platform of Palaeogenetics at the École Normale Supérieure de Lyon (France) and the Archaeological Research Laboratory of Stockholm University (Sweden). After ultra-violet sterilization of the outermost surfaces and removal of an approximately 1 mm layer from the surface, some powder of bone/tooth was collected from each sample using a sterile scalpel or a Dremel tool. Replicas were kept in a freezer at -20°C for subsequent analyses.

3.3. Isolation of aDNA

Bone or tooth powder (100–200 mg) was digested overnight with 1 mL of buffer EDTA (ethylene diamine tetra acetic acid, pH 8) 0.5 M and Urea 1 M with 10 μL of proteinase K (20 mg/mL in water) at 38°C with constant agitation. For each sample, the lysate volume was concentrated down to 100 μL with Amicon columns (Millipore-Amicon Ultra-4 30 k Da) through centrifugation at 4,000 g for 10–15 min. The sample volume was then mixed with $5\times$ PB buffer from the commercial kit QIAquick PCR Purification Kit from Qiagen. Ancient DNA was recovered, following the manufacturer's protocol, in a final volume of 100 μL . Two negative extraction controls were included in every batch of six samples. Duplicates of aDNA extracts were obtained independently from each specimen.

3.4. Mitochondrial DNA amplification

We analysed a *D-loop* fragment of 181 base pairs (bp). In extant dogs, this region is amplified by Polymerase chain reaction (PCR) with DL1-DL2 primers (Leonard et al., 2002), and recovers the majority of the mtDNA haplotypic variability included in the most frequent dog haplogroups (i.e., Hgs A, B, C and D). These primers were also useful in ancient samples of dogs and wolves. We used primer pairs DL1/DL3 and DL7/DL2 (Table 3) in two separate PCR reactions to amplify the mtDNA region between positions 15,495 and 15,676 in the dog reference sequence (EU789784). After amplification, these two fragments of 187 and 108 bp, respectively, superimpose for an extension of 94 nucleotides.

PCR mixtures contained 2 μL of genomic DNA, $1\times$ Taq polymerase buffer (Applied Biosystem), 2.5 mM of MgCl_2 (Applied Biosystem), 0.25 mM of each deoxynucleoside triphosphate (dNTP) (Sigma), 0.02 mg Bovine Serum Albumin (Roche), 0.5 μM of each PCR primer, 2.5 U of Hot start Taq DNA polymerase (Applied Biosystem) and PCR water (Qiagen) in a total of a 25 μL PCR reaction. In all amplification reactions, the primer sequences included multiplex identifiers for libraries (MIDs), i.e., 10 nucleotide sequence tags that allow for multiplexing in emulsion PCR during 454-sequencing (Roche). To monitor for possible aerosol contaminations, a specific control was included in every PCR assay, which were limited to

eight aDNA extracts. The aerosol control was a single tube that was kept opened during the PCR set up. We also monitored possible contaminations from reagents by setting up a negative control with water as a replacement to DNA extract (PCR-mix control). Negative controls were systematically used, and all extraction blanks were also subject to PCR amplification.

Amplification reactions were performed in an Applied Biosystems thermal cycler (Veriti), with an initial denaturation step of 94°C for 10 min; followed by 55 cycles of denaturation at 94°C (30 s), annealing at 52°C (30 s for DL1/DL3 primer pair) or 48°C for (30 s for DL7/DL2 primer pair), and extension at 72°C (45 s); followed by a final extension step at 72°C for 10 min.

Amplifications were verified by the electrophoresis of 5 μL of each PCR product in 2% ethidium bromide agarose gels in Tris/Borate/EDTA (TBE) ($0.5\times$) buffer. Electrophoresis ran at 10 V/cm for 30 min. Samples that provided positive signal at the expected size were sequenced. Negative controls remained blank through agarose gel inspection.

For DNA sequencing, 20 μL of the remaining PCR product were electrophoresed in a second 2% agarose gel and the amplified DNA excised from the gel with a sterile scalpel under UV light. The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen) and eluted in 30 μL elution solution. If primer dimer was present, we used the MinElute PCR Purification Kit (Qiagen) following the manufacturer's protocol.

3.5. Mitochondrial DNA sequencing

We used the Quant-iT™ dsDNA High-Sensitivity Assay Kit (Invitrogen) to quantify the purified positive PCR products. Equimolar mixtures of pooled amplification products were sequenced using the 454-GS Junior technology (Roche) in accordance to the manufacturer's protocols. Sequence reads were identified through primers and MIDs and aligned against reference NCBI sequences. The GALAXY platform (<http://galaxy.psu.edu/>), a web-based genome analysis tool (Afgan et al., 2016) was used to obtain the sequence files for each pooled PCR product. Dog sequences were compared with the DNA sequence information stored in the GenBank database. This was done using the "basic local alignment search tool" (BLAST) program (<http://blast.ncbi.nlm.nih.gov/>) (Altschul et al., 1990) and the blastn algorithm.

For multiple alignments, the Seaview software (Galtier et al., 1996) was used and 60% consensus sequences were generated for each individual from independent amplifications (see sequencing coverage for each dog specimen in Table 4). If any conflict persisted preventing haplogroup assignment the specimen was sequenced until resolution. Partial mtDNA sequences of ancient *Canis* remains were deposited in GenBank (accession numbers KY014672–KY014674; KY014684–KY014894 and KY464999) (Table 4).

3.6. Phylogenetic analyses

Relationships between haplotypes were investigated using NETWORK v5.0.0.0 (Fluxus Technology Ltd, 2004–2016) software

Table 2
Measurements of the humerus distal breadth from various archaeological sites in Portugal and Spain. N – number of specimens measured; Min. – minimum value; Max. – maximum value; SD – Standard Deviation; CV – Coefficient of Variation (%).

Archaeological Site	Location	Country	Roman province	Chronology	N	Min.	Max.	Average	SD	CV (%)	References
20th Century Wolves		Portugal			30	32.4	48.9	41.8	4.2	10.0	(Detry and Cardoso, 2010)
20th Century Dogs		Portugal&Spain			25	19.3	47.5	29.3	7.7	26.3	C. Detry, personal communication
Silos de Carnide	Lisboa	Portugal		Modern period	8	20.9	28.6	24.7	3	12.1	C. Detry and S.J.M. Davis, personal communication
Alcáçova de Santarém	Santarém	Portugal		Modern period	1	22.3	22.3	0.0	0.0	0.0	(Davis, 2006)
Alcáçova de Santarém	Santarém	Portugal		Modern period	9	20.9	28.6	24.4	3	12.3	
Alcáçova de Santarém	Santarém	Portugal		Christian period	1	36.6	36.6	0.0	0.0	0.0	(Davis, 2006)
Alcáçova de Santarém	Santarém	Portugal		Islamic period	1	27.2	27.2	0.0	0.0	0.0	(Davis, 2006)
Els Mallols	Barcelona	Spain	<i>Tarraconensis</i>	Medieval period Late Roman (3rd–4th cent. AD)	2	27.2	36.6	31.7	4.5	14.2	
Odrinhas	Sintra	Portugal	<i>Lusitania</i>	Late Roman (3rd–4th cent. AD)	3	26.1	38.7	33.1	5.3	16.0	(Catagnano, 2016)
Astorga	Léon	Spain	<i>Tarraconensis</i>	Late Roman (3rd–4th cent. AD)	10	20.1	31.6	26.4	3.7	14.0	(Davis and Gonçalves, 2017)
Vila Madrid	Madrid	Spain	<i>Lusitania</i>	Late Roman (3rd–4th cent. AD)	19	23.7	39.5	29.6	4.8	16.2	(Fernandez-Rodriguez, 2000)
Baetulo	Barcelona	Spain	<i>Lusitania</i>	Imperial Roman (1st–2nd cent. AD)	17	21.3	38.4	31.3	4.9	15.7	(Colominas, 2009)
Munigua	Sevilla	Spain	<i>Baetica</i>	Imperial Roman (1st–2nd cent. AD)	1	26.2	26.2	0.0	0.0	0.0	(Colominas, 2009)
Calle Almendralejo	Mérida	Spain	<i>Lusitania</i>	Imperial Roman (1st–2nd cent. AD)	1	20.0	20.0	0.0	0.0	0.0	(Boessneck and von den Driesch, 1980)
Lugo	Galiza	Spain	<i>Tarraconensis</i>	Imperial Roman (1st–2nd cent. AD)	24	20.1	31.6	26.4	3.7	14.0	C. Detry and F.J. Heras, personal communication
Mesas de Castelhino	Almodóvar	Portugal	<i>Lusitania</i>	Imperial Roman (1st–2nd cent. AD)	2	25.6	32.4	29.0	3.4	11.7	(Fernandez-Rodriguez, 2000)
Cerro de la Tortuga	Malaga	Spain		Republican Roman (1st–2nd cent. BC)	1	22.7	22.7	0.0	0.0	0.0	(Valenzuela-Lamas and Fabião, 2012)
Can Xercanvins	Barcelona	Spain		Roman period Iron Age	79	20.0	40.9	29.9	5.3	17.7	
Mas Castellar	Girona	Spain		Iron Age	1	26.2	26.2	0.0	0.0	0.0	(von den Driesch, 1973)
Rua dos Correiros	Lisbon	Portugal		Iron Age	1	34.1	34.1	0.0	0.0	0.0	(Catagnano, 2016)
Cabezo Redondo	Alicante	Spain		Iron Age	5	23.6	33.3	28.5	3.3	11.6	(Catagnano, 2016)
Monachil	Granada	Spain		Iron Age	1	25.4	25.4	0.0	0.0	0.0	(Detry et al., 2016)
Purullena	Granada	Spain		Iron Age Bronze Age	8	23.6	34.1	28.5	3.5	12.3	
Fuente Álamo	Almeria	Spain		Bronze Age	7	24.3	30.6	27.2	1.9	7.0	(Boessneck et al., 1969)
Terrena del Reloj	Granada	Spain		Bronze Age (Argaric)	4	26.5	31.7	28.6	2.0	7.0	(Lauk, 1976)
Muntanya Assolada	València	Spain		Bronze Age	10	22.0	31.0	26.6	2.9	10.9	(Lauk, 1976)
Azuer	Ciudad Real	Spain		Bronze Age	7	26.0	31.0	27.9	1.7	6.1	(von den Driesch et al., 1985)
Can Roqueta	Sabadell	Spain		Bronze Age (Argaric)	2	23.5	29.0	26.3	2.8	10.6	(Milz, 1986)
Cerro de la Encina	Granada	Spain		Bronze Age	1	25.8	25.8	0.0	0.0	0.0	(von den Driesch, 1973)
Zambujal	Torres Novas	Portugal		Bronze Age	1	33.5	33.5	0.0	0.0	0.0	(Boessneck and von den Driesch, 1980)
Leceia	Oeiras	Portugal		Bronze Age	9	25.0	33.0	28.3	0.0	0.0	(Albizuri, 2011)
Cerro de la Virgen	Granada	Spain		Bronze Age	5	25.9	33.2	29.0	2.5	8.6	(Friesch, 1987)
Les Jovades	Alacant	Spain		Bronze Age Chalcolithic	46	22.0	33.5	27.8	2.6	9.4	
Marizulo	Guipuzcoa	Spain		Chalcolithic	1	26.0	26.0	0.0	0.0	0.0	(von den Driesch and Boessneck, 1976)
Camino del Molino	Murcia	Spain		Chalcolithic	3	26.0	29.0	27.5	1.2	4.4	(Pires et al., 2001)
Camino de las Yeseras	Madrid	Spain		Chalcolithic	6	24.8	30.2	27.6	1.6	5.8	(von den Driesch, 1972)
Muge	Salvaterra de Magos	Portugal		Chalcolithic	1	25.8	25.8	25.8	0.0	0.0	(Sanchis and Sarrion, 2004)
				Chalcolithic	1	33.8	33.8	33.8	0.0	0.0	(Altuna, 1967)
				Chalcolithic	40	24.4	38.9	28.2	3.0	10.6	(Catagnano, 2016)
				Chalcolithic	3	28.5	31.5	30.0	1.2	4.0	(Daza, 2015)
				Chalcolithic Mesolithic	55	24.4	38.9	28.2	2.8	9.9	
					1	23.5	23.5	23.5	0.0	0.0	(Detry and Cardoso, 2010)

to construct a Median-Joining (MJ) network (Bandelt et al., 1999). Nucleotide (nt) substitutions weighted 10 for transitions and 30 for transversions so that the component with the lowest expected mutation rate was assigned the highest weight (Bandelt et al.,

2000). The identification of dog clades was undertaken through comparison with reference sequence data of extant Iberian dogs and wolves trimmed to the 181 base pairs fragment.

Identification of dog clades was done by comparison with

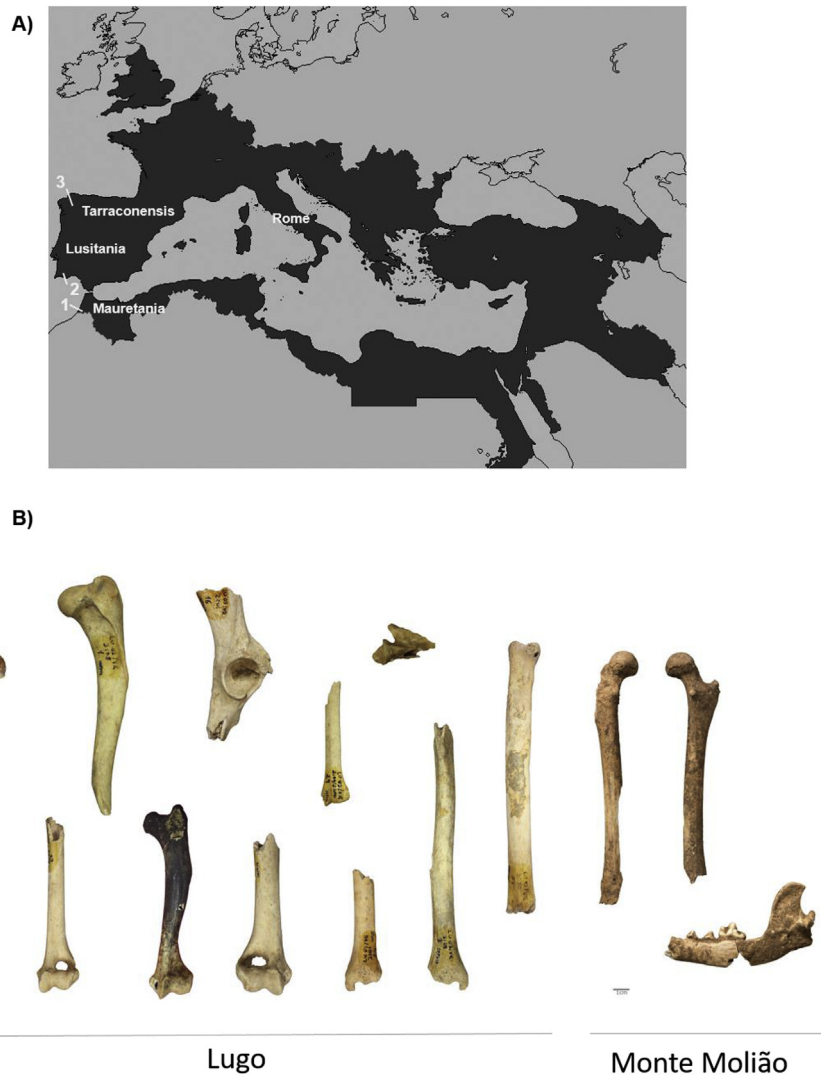


Fig. 2. A) Location of the archaeological sites from where the 15 dog remains used in the genetic analysis were recovered. The sampling was carried out in the western-most range of the Roman Empire, namely (from south to north): 1 - Thamusida, Morocco (*Mauretania Tingitana*); 2 - Monte Molião, Portugal (*Lusitania*); 3 - Lugo, Spain (*Tarraconensis*). Shaded in black is the maximum extent of the Roman empire. B) Sampled materials from Lugo and Monte Molião. Note: The sample from Thamusida was fragmented after sub-sampling for DNA analysis and no picture was taken. Photos by Carlos Fernandez-Rodriguez (material from Lugo) and José Paulo Ruas (material from Monte Molião).

Table 3

Primer sequences for mtDNA-control region amplification in *Canis*.

Primer Name	Primer Sequence 5' – 3'
DL1F forward	GTG CTA TGT CAG TAT CTC CAG G
DL3R reverse	CCC TTA TTG GAC TAA GTG ATA TGC AT
DL7F forward	TAT TAT ATC CTT ACA TAG GAC
DL2R reverse	GCA AGG GTT GAT GGT TTC TCG

reference sequence data on extant dogs from Iberia and North Africa, and comprising breed and village dogs from 1) Pires et al. (2006), 49 haplotypes, 887 base pair long with GenBank accession numbers AY706476–AY706524 (Pires et al., 2006); and 2) Pang et al. (2009), haplotypes A4, A5, A10, A11, A19, A20, A22, A33, A34, A127, B1, D6, D7 (Pang et al., 2009).

An alignment of all these sequences/haplotypes is available from [Partial_MtDNA_SequenceAlignment_DOG_Roman_QInt](#) (fasta).

Short mtDNA fragments may not exactly reflect the phylogeny as determined by complete mitogenomes. We thus constructed a Bayesian phylogenetic tree to confirm haplogroup assignment of

the partial mtDNA sequences obtained in this study by comparison with well-defined dog matrilineages from GenBank. The software MrBayes (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) was used with the evolutionary model GTR + I + gamma. The shape parameter of the gamma distribution was 0.7424 and the proportion of invariable sites (I) was 0.6851. We used 10,000,000 generations, sampling every 1,000th generation, and default settings for the remaining options. Convergence of the Monte Carlo Markov Chain and burn-in were determined through the analysis of the generations versus the log probability plot using the trace analysis tool TRACER v1.6. (Rambaut et al., 2014). The initial burn-in step discarded 20% of the samples. This allowed for haplogroup assignment of all our Roman dog partial mtDNA sequences with high confidence.

4. Results

4.1. Zooarchaeological analyses

Tables 1 and 2 and the associated histograms (Figs. 3 and 4)

Table 4
Details concerning the 15 Roman dog remains examined at the molecular level in this study. PT stands for Portugal, ES for Spain and MA for Morocco. The molecular analyses were performed in two independent laboratories: AFL in Stockholm, Sweden and ENS in Lyon, France.

Sample	Archaeological site, Country	Chronology	Skeletal element recovered	aDNA Laboratory	mtDNA sequence (base pairs)	454 sequencing coverage	GenBank Accession number	mtDNA Haplogroup (Hg)
SEP013	Lugo, ES	3rd - 4th cent. AD	tibia	AFL, Stockholm University, Sweden	139	559	KY014684	A
SEP014	Lugo, ES	3rd - 4th cent. AD	humerus	AFL, Stockholm University, Sweden	139	155	KY014685	A
SEP015	Lugo, ES	3rd - 4th cent. AD	radius	AFL, Stockholm University, Sweden	139	42	KY014686	A
SEP016	Lugo, ES	3rd - 4th cent. AD	scapula	AFL, Stockholm University, Sweden	139	83	KY014687	A
SEP017	Lugo, ES	3rd - 4th cent. AD	pelvis	AFL, Stockholm University, Sweden	139	48	KY014688	A
SEP018	Lugo, ES	3rd - 4th cent. AD	humerus	AFL, Stockholm University, Sweden	139	29	KY014689	A
SEP019	Lugo, ES	3rd - 4th cent. AD	humerus	AFL, Stockholm University, Sweden	139	50	KY014690	A
SEP020	Lugo, ES	3rd - 4th cent. AD	radius	AFL, Stockholm University, Sweden	139	38	KY014691	D
SEP021	Lugo, ES	3rd - 4th cent. AD	tibia	AFL, Stockholm University, Sweden	139	135	KY014692	A
SEP022	Lugo, ES	3rd - 4th cent. AD	femur	AFL, Stockholm University, Sweden	139	126	KY014693	D
SEP023	Lugo, ES	3rd - 4th cent. AD	humerus	AFL, Stockholm University, Sweden	139	23	KY014694	D
LYEP57	Thamusida, MA	1st cent. AD	tooth - lower third pre-molar	ENS-Lyon, France	109	225	KY464999	A
LYEP59	Monte Molião, PT	1st cent. BC	proximal femur	ENS-Lyon, France	181	746	KY014672	A
LYEP60	Monte Molião, PT	1st cent. AD	mandible fragment	ENS-Lyon, France	166	110	KY014673	A
LYEP61	Monte Molião, PT	1st cent. BC	proximal femur	ENS-Lyon	181	479	KY014674	A

summarize information for each archaeological site and its period. Data from the Roman and Late Roman periods—eight archaeological sites for the M_1 length measurements and nine for the humerus distal breadth—confirm the wide variability in size for dogs at that time.

Among the 15 *Canis* specimens from the Roman period selected for aDNA analyses, only three were possible to measure and their measurements fall within the range of M_1 length (one sample, Monte Molião) and humerus distal breadth (two samples, Lugo) for dogs rather than for wolves. Besides this metrical criterion, all archaeological information from where these canid elements were found corresponds to domestic contexts, thus reinforcing their identification as the domestic subspecies.

The results suggest that Chalcolithic and Bronze Age dogs are significantly more uniform than Roman and present-day ones. The low number of measurements for the Iron Age and Modern times prevents us from knowing whether these dog populations were either diverse or homogeneous.

As for cranial variability, the CV calculated for the Roman period (eight distinct archaeological sites) indicates that M_1 length variability in Roman time is larger (CV = 10.8%) compared to other periods (1–7.4%), the same as in Iron Age (CV = 10.8%), but lower when compared to extant dogs (CV = 15.5%) (Table 1). The humerus distal breadth measurements show similar results: the CV calculated from the combined values of nine different archaeological sites show its highest value in the Roman period (CV = 17.7%) followed by extant dogs (CV = 26.3%). For this measurement, the CV value for the Iron Age is much lower (CV = 12.3%).

The stacked histograms (Figs. 3 and 4) also show that the amplitude of values seems higher in the Roman period with smaller dogs observed more frequently.

The results of the Siegel-Tukey test (for assemblages with more than 5 samples) suggest that molar size has greater variability than humerus breadth in different chronological periods (Table 5): 9

comparisons found no significant differences in the diversity of values of humerus breadth, and only one (Chalcolithic vs. Roman) had highly significant differences ($p < 0.01$), compared to 5 comparisons with high significant differences ($p < 0.01$) and 7 comparisons with no significant differences on the first molar. Interestingly, the length of M_1 is more homogeneous in Chalcolithic dogs than in any other periods.

4.2. Genetic analyses

We were able to recover DNA from a total of 15 *Canis* remains, as follows: Thamusida, Morocco (*Mauretania Tingitana*), $n = 1$; Monte Molião, Portugal (*Lusitania*), $n = 3$, and Lugo, Spain (*Tarraconensis*), $n = 11$. None of the controls showed any signs of contamination. Table 4 summarizes the samples analysed for ancient DNA.

Mitochondrial DNA data comprised sequences of 109–181 nucleotides and a total of 17 variable nucleotide sites. We observed five dog mitochondrial haplotypes and detected a single new variant, in Portugal, after comparison with data for extant dogs. These ancient haplotypes could be grouped into two of the major mtDNA lineages observed in extant dogs, i.e., clades A and D (Fig. 5).

More precisely, in Roman times, we detected dog remains carrying haplotypes that belong to mtDNA clades A ($n = 12$: Portugal 3, Spain 8 and Morocco 1) and D ($n = 3$, Spain) (Fig. 6). Interestingly, the older samples from Portugal, which are not contemporaneous with neither African nor Spanish samples, show two different haplotypes within clade A. One sequence (from Monte Molião Portugal, LYEP60) is considered a singleton and is not represented in extant dogs. So far, neither clade C nor B haplotypes were detected in the Roman dogs studied (Fig. 6). In Lugo, northwest Spain, we detect the highest number of haplotypes. These haplotypes differed by up to 7 nucleotides. Evidence for the presence of clade D in the Iberian Peninsula first appears in archaeological dog remains from Roman Spain.

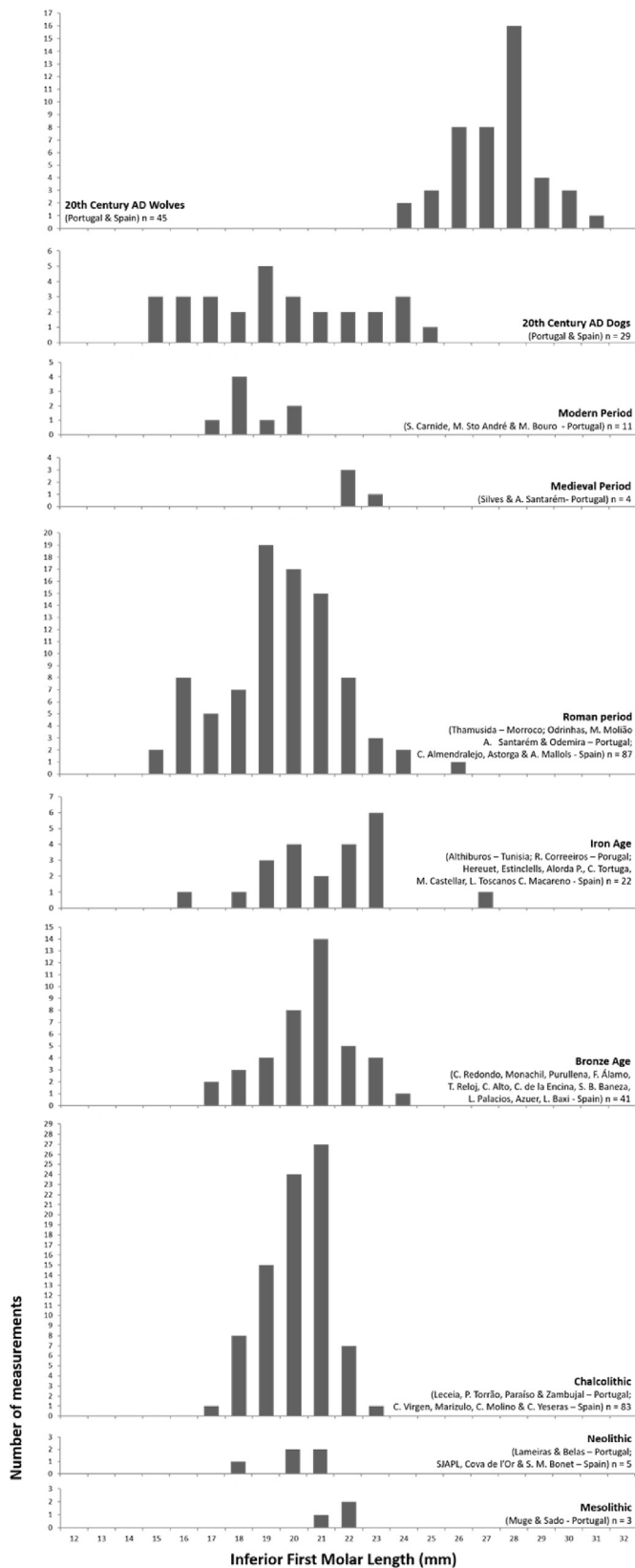


Fig. 3. Stacked histograms with measurements for the length of the lower first molar (mm) of dogs and wolves from the Iberian Peninsula. A diachronic view from several archaeological sites in Iberia and North Africa are compared with measurements from reference skeletal collections (20th century). For more details about these sites and samples see Table 1. [Chalcolithic – 5,000–4,000 years cal BP; Neolithic – 7,500–5,000 years cal BP; Mesolithic – 8,000–7,500 years cal BP].

5. Discussion

Teeth and bones generally preserve well in archaeological sites and can provide information about ancient animals using osteometrical and genetic analyses. The first lower molar generally provides a distinction between dogs and wolves (Tchernov and Valla, 1997; Detry and Cardoso, 2010), and it is a useful element also to assess the degree of variability in size and shape of canid populations.

The lengths of the lower molars (M_1) and the breadths of long bones of the *Canis* remains analysed in this study clearly show the wide variability of dog morphotypes in the Roman period, which include small sized “dwarf dogs”. The variability attested in Roman times is not observed in earlier periods, perhaps with the exception of the Iron Age. Only present-day dogs have a larger variability of sizes and shapes than the Roman ones.

In many organisms, the coefficient of variation (CV) for different traits in natural populations lies between 4 and 10% (Simpson et al., 1960). For the Roman period as well as for Iron Age and extant dogs, the CV is greater than 10%, as shown in the CV of M_1 length and distal humerus breadth (Tables 1 and 2). This is compatible with a high level of phenotypic variability, as a result of a strong artificial selection for these phenotypes indicating that human-directed selection pressure and consequent morphological changes were well established in Roman dogs. Such variability was expected since it has been observed in different areas of the Roman empire, namely in England (Bennett and Robert, 2016), North Africa (MacKinnon, 2010) and Iberia (Boessneck and von den Driesch, 1980; Altuna and Mariezkurrena, 1992; Colominas, 2015). A reduction of the CV after the Roman period also appears to be evident. This can be related to the absence of extreme dogs that only appear again in the 20th century due to artificial selection mainly for aesthetical purposes.

Regarding the genetic analysis, the site of Lugo provided most of the samples analysed ($n = 11$). Thus, in this site, higher mtDNA diversity was observed. Other than the number of samples, the fact that they date from Late Roman times may also have had an influence. To our knowledge, this is the first time that the presence of clade D haplotypes is attested among historic dogs from Iberia. This maternal lineage possibly originates in the Near-East, and has been detected in South-Eastern and Central Europe as early as the Neolithic period (Pionnier-Capitan, 2010). In Iberia, the detection of the first dog D-haplotypes occurs much later. The dog remains from Lugo are dated to Late Roman times—3rd–4th century AD (ca. 1,600–1,700 years ago)—but this haplotype could have come to the Iberian Peninsula any time after the Neolithic, given the continuous human contacts along the Mediterranean since then. This includes early contacts (Davis and Simões, 2016; Isern et al., 2017) as well as the more intense Mediterranean trade in the Iron Age, significantly Phoenicians. This is supported by the presence of numerous colonies and imports along the Southern coast of the Iberian Peninsula and North African (Aranegui, 2016; Aubet, 1987; Ponsich, 1968). First contacts between Iberia and North Africa date of much earlier, at least since the Early Iberian Neolithic (Martínez-Sánchez et al., 2017), probably with a diffusion of farming across the Straits of Gibraltar following a North-to-South direction (from Iberia to North Africa) (Zilhão, 2014). A more comprehensive sampling may reveal an earlier presence of clade D (and eventually other clades) in the Iberian Peninsula and North Africa.

The presence of dogs carrying more distinct mtDNA lineages in Lugo may also be explained by the fact that it was an administratively and politically very important Roman city (Rodríguez Colmenero, 2011). Considering the sites studied here, Lugo was probably the one with more contacts with other parts of the Roman

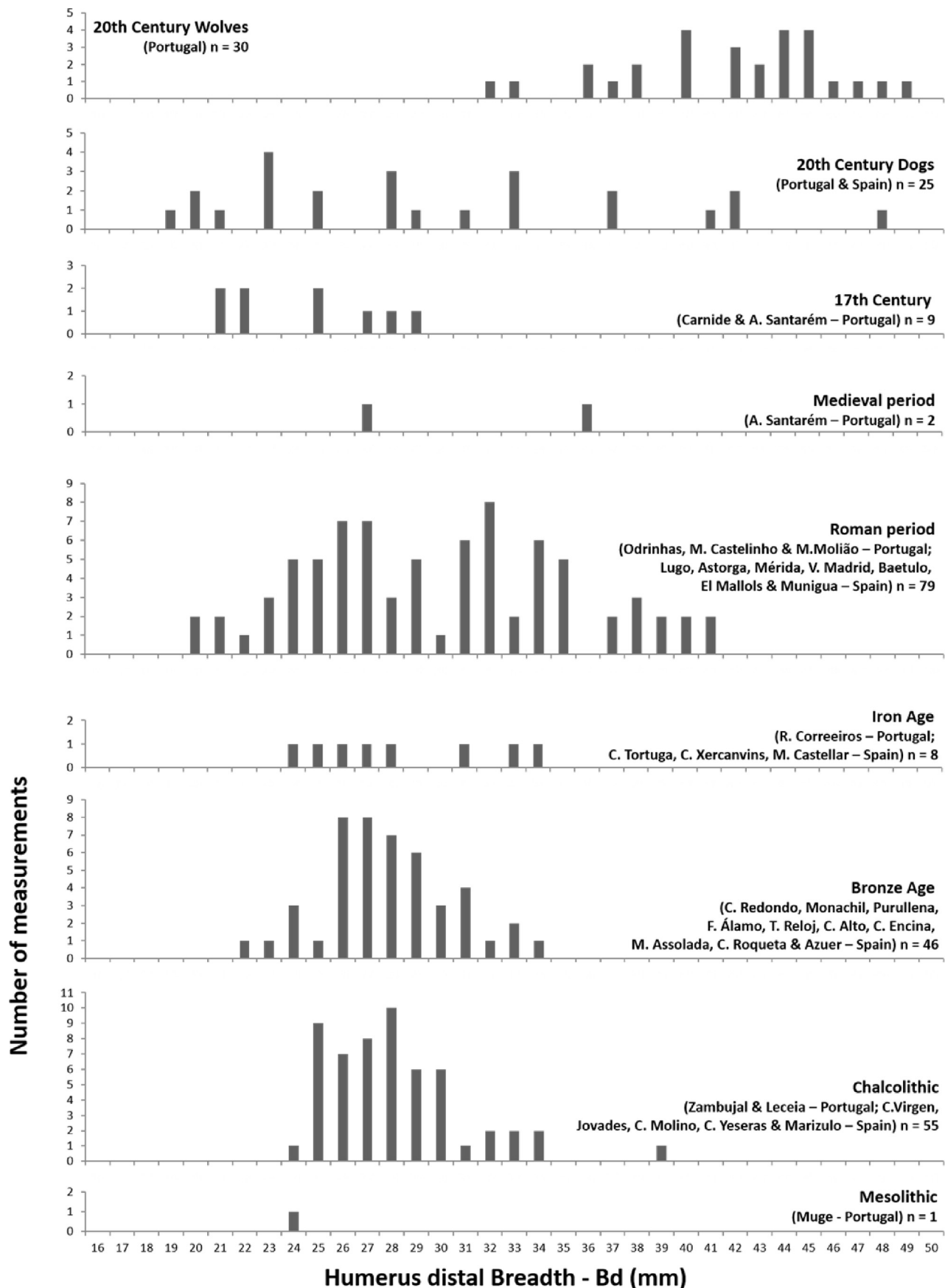


Fig. 4. Stacked histograms with measurements for the humerus distal breadth (mm) of dogs and wolves from the Iberian Peninsula. A diachronic view from several archaeological sites in Iberia and North Africa are compared with measurements from reference skeletal collections (20th century). For more details about these sites and samples see [Table 2](#). [Chalcolithic – 5,000–4,000 years cal BP; Neolithic – 7,500–5,000 years cal BP; Mesolithic – 8,000–7,500 years cal BP].

Table 5

Results of the Siegel-Tukey test (p-values) on the humerus distal breadth (HU Bd) and length of the first lower molar (M1 L) and per assemblage/period. Above diagonal, n.s. stands for non-significant ($p > 0.05$), * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

HU Bd	Chalcolithic	Bronze	Iron	Roman	Modern	Present-day	Number of measurements
Chalcolithic		n.s.	n.s.	**	n.s.	*	55
Bronze	0.9375		n.s.	*	n.s.	*	46
Iron	0.2876	0.3804		n.s.	n.s.	*	8
Roman	0.005013	0.02842	0.3702		n.s.	*	79
Modern	0.1067	0.1327	0.5966	0.7203		n.s.	9
Present-day	0.01566	0.04019	0.03377	0.029	0.2261		25
M1 L	Chalcolithic	Bronze	Iron	Roman	Modern	Present-day	Number of measurements
Chalcolithic		n.s.	**	**	**	**	83
Bronze	0.2019		*	n.s.	n.s.	**	41
Iron	0.001734	0.03958		n.s.	n.s.	n.s.	22
Roman	0.001105	0.3372	0.1661		n.s.	*	87
Modern	0.00929	0.221	0.4914	0.7114		*	11
Present-day	0.009162	0.003261	0.09402	0.02602	0.03784		29

Empire. Additionally, the detection of the less frequent clade D may echo the consolidation of distinct dog types, as we describe herein for this period. Our results show the presence and breeding of dogs of distinct mtDNA haplogroups, A and D, in Iberia continuously for over 1,600 years.

Samples from Monte Molião, dated to the 1st cent. BC - 1st cent. AD (ca. 2,000 years ago), also reveal interesting aspects of the genetic composition of dog populations in the past. The two mtDNA haplotypes genotyped are not shared with the other dog samples analysed, and one of them consists of a new variant not found in extant dogs. This suggests some level of mtDNA diversity in dogs at this site and that some dog-lineages may have been lost through time. However, we cannot completely discard the possibility that the nucleotide differences found between these single haplotypes and the others results from some level of DNA degradation. In particular, the Monte Molião LYEP60 singleton (a mtDNA sequence found only in one individual), is defined by a nucleotide position that exhibits a thymine (T) while all others exhibit a cytosine (C), which could be a result of damage. In this case, thymine was the prevalent nucleotide in 79 out of the 110 reads for this sample.

The mtDNA sequence of the African dog specimen belongs to clade A, which is nowadays the most common lineage found in dogs worldwide, including North Africa (Boyko et al., 2009) and Iberia (Pires et al., 2006). Here we contribute only one sample from Thamusia: interestingly, it shared a clade A haplotype with three other samples from Lugo. This result may indicate that either this was a common lineage in each of these regions or the existence of gene flow between dogs from these regions. Dogs could have been easily transported across the Mediterranean by humans following maritime trading routes. There is considerable evidence for maritime trade between North Africa and North Western Iberia in Roman times (e.g. Naveiro López, 1991), that became even more consolidated in the Late Roman period (e.g. Fernández Fernández, 2014). However, the terrestrial routes could also have been relevant, namely through *via de la Plata* (linking *Augusta Emerita* – Mérida with *Asturica Augusta* – Astorga) and *via XIX* (linking *Bracara Augusta* – Braga with *Asturica Augusta* through *Lucus Augusti* (Lugo)) (De Soto, 2013).

Despite its geographic proximity, the Thamusia sample does not reflect evidence for gene flow between dogs from this region and Monte Molião (no haplotype sharing). This is consistent with other archaeological materials (e.g. pottery), that suggest that, during the Republican and the Imperial Roman periods, imports at Monte Molião from North Africa mainly originated in the

province of *Africa proconsularis* (present-day Tunisia) (Arruda, 2007; Arruda and Sousa, 2013). Nevertheless, this may also be related to the limited sampling in both sites and possible genetic drift.

Fig. 1, as well as data from Bennett and Robert (2016), show that Roman iconography reflects a large amount of morphological variability in dogs. The Roman mosaics from Conímbriga, a very important city in Roman *Lusitania* (Portugal), show several dog coat colours and even different shapes of the cranium (Fig. 1). These are probably accurate representations of Roman dogs, although we cannot be certain about their geographical origin. The artists that produced these mosaics could be itinerant specialists inspired by local or foreign models. In Conímbriga there are evidences that the artisans are local and the mosaics with the dogs are described as being depictions of the servants of the owner of the house (Correia, 2013). Additionally, various shapes of dogs are represented in ceramic decorations of the *sigillata* type found in the Iberian Peninsula (Bennett and Robert, 2016; Bustamante and Detry, 2017). However, taken together, iconographic, zooarchaeological and ancient DNA data, all coincide in reflecting the existence of different dog sizes, shapes and origins in Roman times.

6. Conclusion

This study confirms the utility of combining morphological, palaeogenetic and archaeological data to describe past phenotypic and genetic variability of dogs. We detected the presence of various mtDNA lineages during Roman times in Iberia and North Africa, which is in agreement with the documented high morphological diversity of dogs in the iconographic evidence—pottery and mosaics—from these areas. The zooarchaeological study also revealed that the diversity of morphologies was greater in Roman times compared to any other period, with the exception of present-day dogs. This suggests that artificial selection for morphologic traits was well established in Roman times and included lap dogs.

While mtDNA analysis is informative to investigate genetic affinities and demographic changes in domestic animal populations, autosomal DNA (i.e., markers linked to phenotypic traits) is needed to recover signatures of post-domestication changes in inheritable traits due to human selection. Subsequent genetic analysis of the dog specimens described in this study using such markers will allow us to describe some phenotypic traits of Roman dogs for a more comprehensive interpretation of their variability.

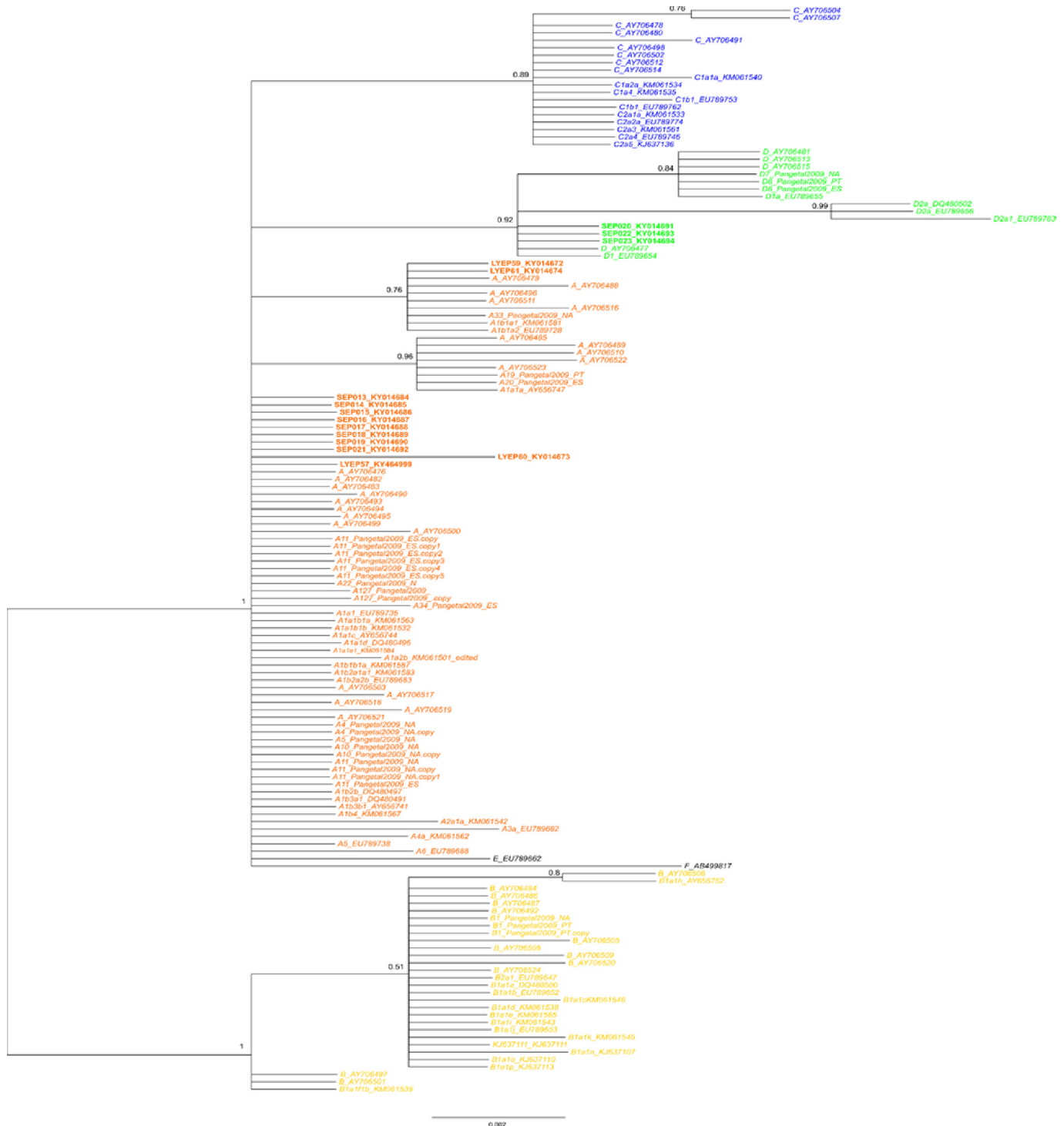


Fig. 5. Bayesian phylogenetic tree used for haplogroup assignment of the 109–181 bp mitochondrial DNA sequences obtained in this study by comparison with well-defined dog matrilineal sequences from GenBank. Sequences from ancient Iberian *Canis* remains are depicted in bold. Extant dog data from GenBank include the following: 54 sequences trimmed from mitogenome data (Duleba et al., 2015), haplogroup/sub-haplogroup nomenclature precedes accession number information; 49 sequences from modern Iberian native breeds and Iberian village dogs (Pires et al., 2006), with haplogroup letters, accession numbers and haplotype numbers shown; 4 Portuguese, 10 Spanish and 14 North African dogs (Pang et al., 2009), with haplogroup letters and haplotype numbers also indicated. Major haplogroups are coloured as follows: A-orange; B-yellow; C-blue; and D-green. Bootstrap values are indicated for each node. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

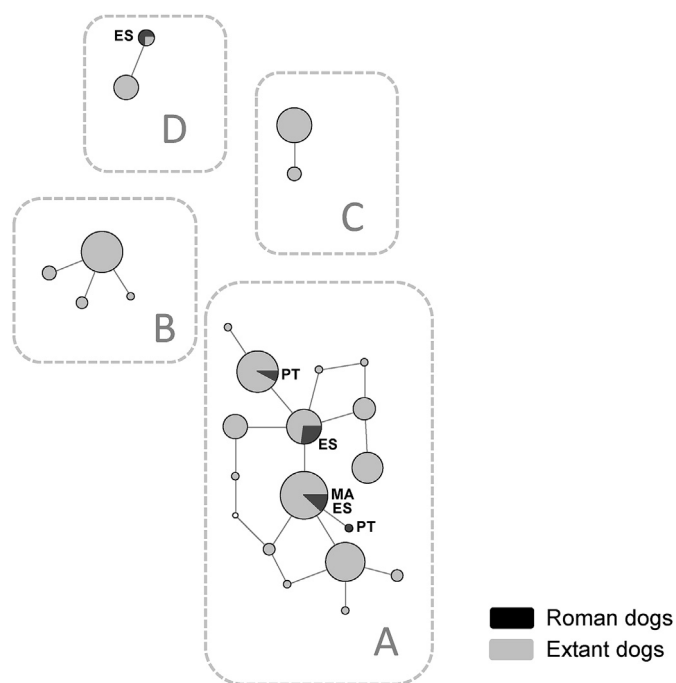


Fig. 6. Median-joining network of mtDNA sequences. Clades A, B, C and D represent the major haplogroups found in extant dogs as defined by mitogenomes in Duleba et al. (2015). Five distinct ancient haplotypes were recovered from Roman dog samples (black) and which clustered with haplotypes A and D of extant dogs (gray). ES refers to haplotypes found in Roman Spain, MA in Morocco and PT in Portugal. The single white dot represents a median vector generated by the software to indicate an expected but unsampled haplotype.

Conflicts of interest

The authors declare no conflicts of interest.

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