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Ancestral matrilineages and mitochondrial DNA diversity of the Lidia cattle breed

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Summary

To clarify the genetic ancestry and the mitochondrial DNA (mtDNA) diversity of the Lidia cattle breed, a 521-bp D-loop fragment was sequenced in 527 animals belonging to 70 herds distributed across 29 lineages. The mtDNA diversity recorded was similar to that seen for Middle Eastern breeds and greater than that recorded for the majority of European breeds. Haplotype T3 was the most common (81%), followed by the African T1 haplotype (17%); very low frequencies were recorded for haplotypes T and T2. The results agree with there being two major ancestral lines for the Lidia breed, European and African, similar to that seen for other Mediterranean breeds. A wide range of variation in haplotype frequencies was seen between the examined lineages. Haplotype T3 was present in all those analysed; in five it was the only one present, and in only one lineage (Miura) was its frequency lower than that of T1. T1*, a haplotype reported in Criollo breeds and to date in only a single European breed (the Retinta breed from Spain), was found in a single animal belonging to the Concha y Sierra lineage. Network analysis of the Lidia breed revealed the presence of two major haplotypes: T3 and T1. The Lidia breed appears to be more closely related to prehistoric Iberian and Italian than to British aurochs.

Keywords aurochs, bovine, fighting bull, maternal lineage, mitochondrial DNA.

Introduction

The analysis of mitochondrial DNA (mtDNA) sequence diversity has provided useful information on the origin and diversification of current cattle populations (Loftus *et al.* 1994; Bradley *et al.* 1996; Troy *et al.* 2001). Wild aurochs were domesticated some 12 000 years ago across Europe, Asia and Africa, and the mitochondrial signals of these animals can be seen in modern cattle breeds (Loftus *et al.* 1994; Troy *et al.* 2001; Edwards *et al.* 2004). Five main mitochondrial haplotypes have been described, T, T1, T2, T3 and T4, which show different geographical distributions (Cymbron *et al.* 1999; Troy *et al.* 2001). Haplotype T3 is clearly predominant in central and northern European breeds, while T1 is mainly seen in African breeds (Beja-Pereira *et al.* 2006). Mediterranean breeds have both the T3 and T1 (at a lower frequency) haplotypes because of African influence. Haplotypes T and T2 appear in the breeds of Europe and Africa but at very low frequencies (Bradley *et al.* 1996; Cymbron *et al.* 1999; Troy *et al.* 2001), and T4 is found in Asian breeds (Mannen *et al.* 2004). A new haplotype, T1* (defined as T1 with mutations at positions 16053, 16122, 16139 and 16196) has been reported in Criollo breeds and (to date) in a single European breed (the Retinta breed from Spain) (Miretti *et al.* 2002, 2004) (Table S1).

The relationships between wild aurochs and domesticated cattle breeds, however, remain unclear (Vilà *et al.* 2005). The first auroch mtDNA sequences, collected in Great Britain, typed far from those of modern cattle breeds, suggesting little auroch introgression (Troy *et al.* 2001). Later, however, more ancient auroch sequences from Italy and from the Bronze Age of the Iberian Peninsula revealed haplotype distributions similar to those of modern European cattle breeds (Anderung *et al.* 2005; Beja-Pereira *et al.* 2006). Only one Iberian sample appeared more closely related to the British auroch sequences (Anderung *et al.* 2005). Thus, the introgression of auroch mtDNA into modern cattle breeds has taken place, but it is not clear to

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what degree or whether this varied depending upon geographical location.

Three waves of migration from the original area of cattle domestication arrived at the Iberian Peninsula, influencing the then-current cattle breeds. Two of these migrations followed the route from the Middle East via the European mainland or Mediterranean coast, while the third took the African route; this was possible because of the proximity between Iberia and Africa (Cymbron *et al.* 1999; Anderung *et al.* 2005). African populations have entered the Iberian Peninsula since the Bronze Age (Bogucki 1996; Gkliasta *et al.* 2003). Thus, Spanish cattle breeds may show the northern or southern European haplotype pattern depending on their origin.

The Lidia breed, otherwise known as the fighting bull, has a number of peculiarities that make it one of the most successful of domestic breeds in Spain. It has been spread throughout this country, Portugal and southern France, as well as throughout numerous Central and South American countries (http://www.toroslidia.com/). The first known reference to a Lidia herd is found in the 14th century literature of the kingdom of Navarra: ''Joan de Gris, breeder of Tudela, sent in 1388 two bulls to Pamplona by order of King Charles III 'The Noble' for the fair, in honor of the Duke of Borbon'' (Larrea *et al.* 2005). Between the eighth and 15th centuries, a constantly changing no-man's land formed the frontier between the Muslim and Christian regions of the Iberian Peninsula, favouring the existence of a semi-feral bovine population. The most feral, aggressive animals were commonly selected for fiestas.

During the 18th century, a certain amount of breeding specialization took place, and animals from the traditional herds, which mainly produced meat, were selected to produce animals for these social events. Different types of traditional spectacle demanded different types of behaviour on the part of the bull, which largely explains why this racial group has become fragmented into small lineages, traditionally called *encastes* (Boletín Oficial del Estado 2001), with different levels of gene flow among them. As a consequence, the Lidia breed shows great phenotypic (morphological and behavioural) heterogeneity among its lineages (Boletín Oficial del Estado 2001). The present-day representatives of these lineages are now mainly found in the west and southwest of the Iberian Peninsula, and the lineages do not follow any particular distribution pattern across the geographical distribution areas.

The Lidia breed has likely been subjected to two major historical influences, one exerted by cattle that accompanied the Celtic invasions, and one exerted by African populations, a consequence of geographic proximity (http://www.toroslidia.com). However, the few DNA studies performed have been insufficient to corroborate this idea, and none have examined mtDNA.

Despite the peculiarities described above and the great genetic diversity manifested in its nuclear genetic material (Cañón *et al.* 2008), little is known about the genetic influences that helped form the Lidia breed. The aim of the present work was to investigate these influences by examining the mtDNA of its lineages.

Materials and methods

Five hundred and twenty-nine blood samples were collected from the animals bred in Spain and maintained in a DNA preservation buffer (Dunner & Cañón 2006) until use. Table S2 shows the number of herds and unrelated animals sampled from each lineage. DNA was extracted by standard methods (Sambrook *et al.* 1989). A 521-bp fragment of the mtDNA D-loop was sequenced, encompassing positions 16019–16201 (Anderson *et al.* 1982). Two overlapping D-loop fragments were amplified using PCR. Primer sequences were designed using the software PRIMER version 0.5 (GCG software package). The primers used for the first fragment were DLOOPF 5′-TATGCCCCATGCATATAAGC-3′ and DLOOPR 5′-AAGAAAGAACCAGATGCCTG-3′; the primers used for the second fragment were MITFOR 5′-ATCCCTCTTCTCGCTCCG-3′ and MITREV 5′- TTATGTCCTGTGACCATTGACTG-3′. The amplified fragments were purified using the Concert Rapid PCR Purification System (Life Technologies) according to the manufacturer's instructions. Sequencing was performed using primers DLOOPR and MITREV in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequence alignment was performed using free software available at http://bioinfo.hku.hk/EMBOSS/ (the European Molecular Biology Open Software Suite). The mean number of pairwise differences (MNPD), nucleotide diversity, F_{ST} distances, and Fisher's exact test were obtained using MEGA software v2.1 (Kumar *et al.* 2001) and the ARLEQUIN package v3.11 (Excoffier *et al.* 2005, http://cmpg.unibe.ch/software/arlequin3). Reduced median networks (Bandelt *et al.* 1999) were generated with the resultant sequences and previously published *Bos primigenius* sequences (Troy *et al.* 2001; Anderung *et al.* 2005; Beja-Pereira *et al.* 2006) using the NETWORK 3.0 program (http://www.fluxus-technology.com/). Comparisons were restricted to the region of overlap between the Lidia and *Bos primigenius* D-loop mitochondrial sequences, between positions 16042–16280 (Anderson *et al.* 1982). Partitioning of the total genetic variance into components because of inter-lineages, inter-breeders and inter-individual differences was carried out using the analysis of molecular variance (AMOVA) program implemented in the ARLEQUIN package.

Results

The 529 sample sequences revealed 121 haplotypes, defined by 73 polymorphisms (65 transitions and eight transversions). A total of 53 haplotypes were unique, while the most common haplotypes were shared by 65 animals (frequency

of 12%). Six new mutations of the Old World haplotypes were detected (Table S1) at low frequencies (ranging from 0.2% to 1.3%) (EU373501–EU373506). The haplotype distribution for the Lidia breed as a whole showed a typical southern European pattern, with T3 the predominant haplotype (87%), T1 less common (17%), and T and T2 at very small frequencies (1.2% and 0.6% respectively) (Table 1). The frequency of the T1 haplotype ranges from 5% to 30% in different Mediterranean breeds (Beja-Pereira *et al.* 2006). In the Concha y Sierra lineage, one animal showed the Criollo haplotype (T1*); this has previously been described in just one European breed (Retinta from Spain) (Miretti *et al.* 2002, 2004).

All the lineages investigated showed the T3 haplotype; in five it was the only one present, and only in the Miura lineage did its frequency fall below 50%. Haplotype T1 was found at different frequencies in 24 of the 29 lineages examined, ranging from 3% in Atanasio Fernández to 57% in Miura. Haplotype T2 was present in only two lineages and haplotype T in five, both at very low frequencies (Table 1). Fisher's exact test ($P = 0.006$) confirms the lack of randomness in the differences between lineages of the haplotype frequencies.

The mean MNPD value was 3.7, ranging between 2.3 in Antonio Pérez (which only showed haplotype T3) and 5.5 in Concha y Sierra (the only lineage to show four of the six haplotypes known to date; Table 1). The nucleotide diversity for the entire Lidia breed was 0.74%, varying from 0.4% in Antonio Pérez to 1% in Concha y Sierra (Table 1).

The pairwise F_{ST} values ranged from 0% to 30% (Pedrajas and Miura respectively), 74% of which were significant ($P < 0.05$). The average F_{ST} distance between each lineage and the others showed marked differences, ranging from 2.7% for Braganza to 17% for Pedrajas. Six lineages (Table 2) showed F_{ST} values of >10%, indicating their reproductive isolation and strong differentiation. Six other lineages showed F_{ST} values of <5%, indicating just the opposite. The remaining lineages showed intermediate situations, with F_{ST} values ranging from 5% to 10%. These high F_{ST} values are reflected in the high proportion of the

Table 1 Mean number of pairwise differences (MNPD), nucleotide diversity and frequencies of the main haplotypes for each lineage.

Lineage	MNPD[1]	Nucleotide diversity[2]	Haplotypes (%)				
			T3	T1	T	T2	T1*
Pedrajas	2.6	0.005	100				
Antonio Pérez	2.3	0.004	100				
Urcola	3.4	0.007	100				
Pablo Romero	3.8	0.007	100				
María Montalvo	2.6	0.005	100				
Atanasio Fernández	2.7	0.005	95	2.5	2.5		
Baltasar Ibán	2.5	0.005	93	7			
Vega Villar	4.1	0.008	92	8			
Félix Gómez	4.0	0.008	87	13			
Torrestrella	3.3	0.006	87	13			
Veragua	3.9	0.007	86	14			
Juan Pedro Domecq	3.7	0.007	85	15			
Gamero Cívico	3.2	0.006	83	17			
Conde Santa Coloma	3.8	0.007	81	15	4		
José Marzal	3.9	0.008	80	13	7		
Arauz de Robles	4.1	0.008	80	20			
Contreras	3.4	0.007	80	7		13	
Marqués de Albaserrada	4.6	0.008	80	20			
Conde de la Corte	4.6	0.009	79	21			
Marqués de Villamarta	3.9	0.008	77	23			
Saltillo	3.6	0.007	75	25			
Carlos Núñez	3.4	0.007	75	20	5		
Hidalgo Barquero	3.6	0.007	71	29			
Braganza	3.9	0.008	71	29			
Murube	3.7	0.007	68	32			
Cuadri	3.6	0.007	67	33			
Manuel Arranz	5.0	0.010	64	29	7		
Concha y Sierra	5.5	0.011	56	31		5.5	5.5
Miura	4.0	0.008	43	57			
Average values	3.7	0.007	81	17	1.2	0.6	0.2

[1]Standard errors ranged from 1.3 to 2.8.
[2]Standard errors ranged from 0.003 to 0.006.

Table 2 Average F_{ST} value for each lineage with respect to the others (descending order).

Lineage	F_{ST} distances (%)
Pedrajas	16.7
Miura	14.5
Concha y Sierra	12.7
Baltasar Ibán	12.5
María Montalvo	11.7
Urcola	9.6
Antonio Pérez	9.1
Vega Villar	8.5
Félix Gómez	8.1
Atanasio Fernández	8.0
Manuel Arranz	7.6
Contreras	7.6
Veragua	7.0
Pablo Romero	7.0
Cuadri	6.9
Arauz de Robles	6.8
Torrestrella	6.8
Hidalgo Barquero	6.3
Gamero Cívico	6.0
Murube	5.9
Conde de la Corte	5.8
Saltillo	5.5
Juan Pedro Domecq	4.5
Carlos Núñez	4.4
Marqués de Villamarta	4.3
Conde Santa Coloma	4.2
Marqués de Albaserrada	3.9
José Marzal	3.5
Braganza	2.7

Table 3 Partitioning of the genetic variability among the different sources of variation.

Source of variation	Percentage of variation	Fixation indices
Among lineages	1.2	F_{SC} = 0.10
Among breeders within lineage	10.1	F_{ST} = 0.11
Within breeders	88.8	F_{CT} = 0.01

total variance explained by the genetic differences among lineages obtained by the AMOVA analysis (Table 3).

Figure 1 shows the phylogeny network for the 121 haplotypes (Bandelt *et al.* 1999). Two main clusters can be recognized corresponding to the European haplogroup (T3 plus T and T2), and the African haplogroup (T1). Both networks show a star-like skeleton; the centre of the star corresponds to the haplotype with the highest frequency, while a number of peripheral haplotypes decoalesce. A similar network figure has been observed for Mediterranean breeds (Anderung *et al.* 2005). As described in previous studies, the T1* haplotype appeared in the African cluster but clearly separated from the centre.

The status of the six new haplotypes (Table S1) is dependent on nucleotide 16113. Those with the same nucleotide as haplotype T1 fall into the African cluster, while the rest fall into the European cluster.

When the auroch sequences from Great Britain, Italy and the Bronze Age Iberian samples were added to the network analysis, the topology showed four clusters (Fig. 2). The European and the African clusters still showed a star-like skeleton network. The British

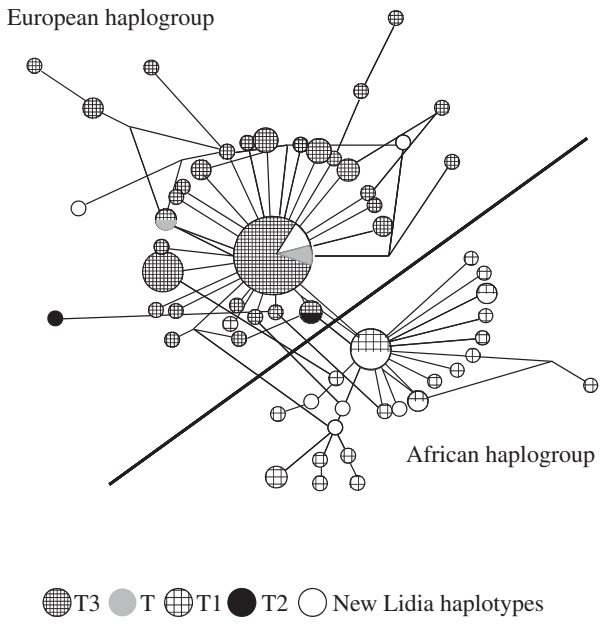

Figure 1 Reduced median-joining network (Bandelt *et al.* 1999) constructed from 121 Lidia breed haplotypes. Circle size is proportional to sequence frequency.

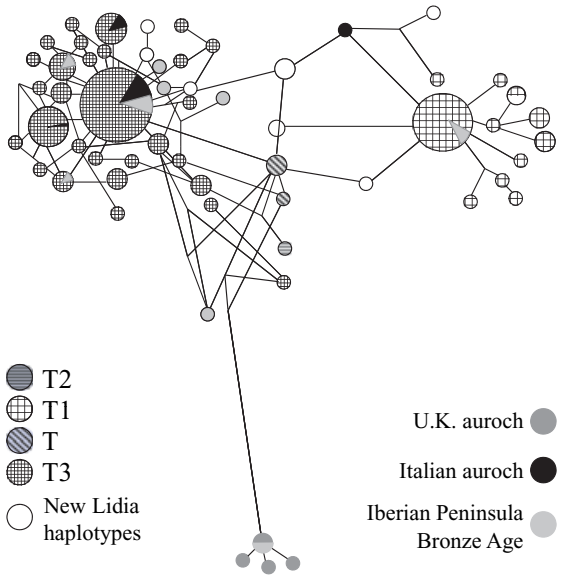

Figure 2 Reduced median-joining network (Bandelt *et al.* 1999) constructed from 121 Lidia breed haplotypes and auroch (*Bos primigenius*) samples from the UK (12300–11000 BCE), Italy (17100–11420 BCE) and the Iberian Peninsula (2670 BCE–1500 CE). Circle size is proportional to sequence frequency.

sequences and one Iberian sample formed another cluster, clearly isolated from the others. Finally, the fourth cluster was located between the European and African clusters, representing the T haplotype and the six new haplotypes found for the Lidia breed. This caused the loss of the star-like skeleton network, giving rise to a dispersed cluster in which no single sequence predominated. The Italian and all but one of Iberian auroch samples were represented in the T3 and T1 clusters.

Discussion

It is remarkable that the average MNPD for the Lidia breed is markedly higher than those of other European (1.8) or African breeds (2.1), but close to those of Middle Eastern (4.0) and Anatolian breeds (3.7) (Bradley *et al.* 1996; Troy *et al.* 2001; Carvajal-Carmona *et al.* 2003). The reduced diversity of European domestic cattle compared with those of the Middle East is consistent with the origin of these animals in the latter region. Nevertheless, it is unusual that a European breed should show an index of diversity similar to that of breeds from Anatolia and the Middle East. Analogous evidence can be obtained from the Lidia breed's nucleotide diversity; the value for the whole breed (0.74%) is higher than those of other European breeds, which range from 0.11% to 0.57% (Loftus *et al.* 1994). This may have two explanations: (i) the geographical location of the Iberian Peninsula and the three influences exerted during the breed-making process (the African, Mediterranean and mainland European influences) could have left the Lidia

breed in possession of all four major Old World haplotypes and (ii) the division of the breed into lineages with different levels of reproductive isolation may have favoured genetic differentiation, thus maintaining the breed's ancestral genetic diversity.

A high level of mitochondrial diversity was also seen within the different lineages studied. The lowest MNPD value, of Antonio Perez (2.5), is higher than that of most European breeds. Only five lineages had an MNPD value of <3. In three of these, only the T3 haplotype was present, and in the other two, it had a frequency of >93%. Eight lineages had an MNPD value of >4, and with the exception of Vega Villar, the remainder showed a relatively high frequency for the T1 haplotype (>10%). The Pearson coefficient correlation between nucleotide diversity and the MNPD value was 0.99%.

Diversity was particularly high in the Concha y Sierra lineage (MNPD = 5.2; nucleotide diversity = 1.1%), in which three of the four Old World haplotypes were present. The identification of a T1* haplotype in a Concha y Sierra animal is remarkable; in Europe it was previously known only in the Spanish Retinta breed (Miretti *et al.* 2002, 2004; Liron *et al.* 2006). This finding is consistent with the presence of this lineage in southern North America and in Central America (a fact confirmed by historical documents). The presence of the T1* haplotype in the Concha y Sierra lineage may be explained by crosses in this area of the Concha y Sierra lineage with Criollo breeds previously introgressed with African zebu, the original carrier of the T1* haplotype (Liron *et al.* 2006).

The investigation of the nuclear DNA of the studied lineages has shown them to have a high degree of genetic differentiation (Cañón *et al.* 2008). The present work with mtDNA confirms this; indeed, and as opposed to what usually happens in domestic bovine breeds (Robertson 1953), the proportion of genetic variability caused by lineages (~11%) and even the genetic differentiation among breeders within lineages (~10%) were high (Table 3). It should be remembered, however, that the present results correspond to one single breed divided into several lineages – comparisons with the results obtained in studies involving several breeds should therefore be undertaken with caution. Among the lineages with the highest F_{ST} values (F_{ST} > 9%), two groups can be identified. The first includes lineages with only one Old World haplotype, or with several but with one at a high frequency plus a lower MNPD value (e.g. Pedrajas, Baltasar Iban, Antonio Pérez, María Montalvo and Urcola). In fact, the common practice of mating related animals with the objective of fixing desirable behavioural traits increases the effect of genetic drift, with genetic diversity becoming lost but the isolation between lineages increasing. The lineages of the second group (Miura and Concha y Sierra) have distinctive features that explain their genetic differentiation from the rest. Concha y Sierra show three distinct Old World haplotypes plus T1*, while Miura is the only lineage with a T1 frequency higher than that of T3.

Two major influences on the Lidia breed have been described in historical documents, one exerted by the cattle that came with Celtic invaders, and the other by African populations, a consequence of their geographic proximity (http://www.toroslidia.com). Certainly, the present network skeleton analysis reveals the presence of two main matrilineages corresponding to European (T3) and African (T1) influences; this reinforces the idea that the Lidia breed is the result of a mixing with animals that arrived in two independent migration waves, one from Europe and one from Africa. The pattern shared by many European breeds, in which haplotype T3 is clearly more abundant than T or T2, confirms the influence exerted on the Lidia breed by domestic cattle originally from the Middle East that arrived in the Iberian Peninsula via the European mainland or Mediterranean route.

Some authors indicate that contemporary Iberian breeds are more closely related to African cattle than to European cattle because of gene flow from Africa in the 1960s and 1970s (Anderung *et al.* 2005). However, the Lidia herdbook indicates this as unlikely. Thus, the influence on the Lidia breed exerted by the African cattle might be traced back to the Muslim/North African Berber expansion (Cymbron *et al.* 1999) – or even earlier, as a recent study of a T1 haplotype in Iberian Bronze Age samples (Anderung *et al.* 2005) suggests.

European aurochs were probably strongly geographically structured, as the analysis of British and Italian aurochs indicates. However, a certain level of spreading may also have been possible given the finding in Spain of a Bronze Age sample with similarities to British aurochs. As expected, a network analysis including samples from prehistoric wild aurochs and prehistoric, domestic, Iberian samples showed more genetic affinity between the Lidia breed and Italian or prehistoric Iberian aurochs than with British aurochs.

One of the six new haplotypes for the Lidia breed is the same as that seen in an Italian auroch sample (haplotype 5 in Table S1). Nevertheless, its frequency in the Lidia breed is clearly lower than that in other Iberian breeds and closer to the frequencies of Balkan or mainland European breeds. It should be remembered that the few auroch samples analysed may not reflect the real distribution of auroch haplotypes. In addition, the similar skeleton networks shown by the Iberian Bronze Age samples and the Lidia breed indicate that no great changes have occurred in the mtDNA of the Lidia breed since the Bronze Age.

In conclusion, the high mtDNA diversity observed in the Lidia breed – closer to that seen in Middle Eastern than European populations – is evidence of a certain degree of primitivism. In fact, the Lidia breed was found to have all the Old World haplotypes plus T1*. The multiple influences on this breed throughout history and its division into

isolated reproductive lineages might explain the high level of mtDNA diversity seen.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Mitochondrial DNA D-loop sequence variation between the six major haplotypes (T, T1, T2, T3, T4 and T1*) and the six new haplotypes for the Lidia breed (1–6) and their frequencies (%).

Table S2 Names of the sampled lineages, number of sampled herds and number of samples from each lineage.

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