Mitochondrial sequence variation suggests an African influence in Portuguese cattle

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A total of 49 samples from indigenous Portuguese cattle breeds were analysed for sequence variation in the hypervariable region of the mitochondrial DNA D-loop. Sequence comparison and phylogenetic analyses revealed that haplotypes fell into two distinct groups. These corresponded with two separate haplotype clusters into which, respectively, all African, or alternatively all sequences of European origin, have previously been shown to fall. Here, the majority of sequences of African type were encountered in three southern, as compared to three northern breeds. This pattern of African influence may reflect an intercontinental admixture in the initial origins of Iberian breeds, or it is perhaps an introgression dating from the long and influential Moorish occupation of the south of the Iberian peninsula.

Keywords: mtDNA; domestication; molecular evolution; population genetics; *Bos taurus*; Portugal

1. INTRODUCTION

Comparisons of mitochondrial DNA (mtDNA) D-loop sequences in cattle population samples of wide provenance have been a valuable source of archaeological inference about the origins and nature of the domestication process (Bradley *et al.* 1998). Initially, Loftus *et al.* (1994) identified a separation of bovine diversity into two mtDNA sequence clusters, which were representative of ancestral *Bos indicus* (zebu) and *Bos taurus* (taurine) lineages. A calibration of the divergence between these yielded an estimate of several hundred thousand years for the common ancestry of the two strains, which was clearly inconsistent with a common domestic origin for all cattle of only 10 000 years BP. It seems likely that zebu cattle may have been domesticated originally from wild oxen (*Bos primigenius namadicus*) in the Baluchistan region (in modern Pakistan) and taurine animals from separate strains of aurochs (*B. p. primigenius*) by the early agricultural societies of the Near East and perhaps also Africa (Meadow 1993; Bökönyi 1976; Wendorf & Schild 1994).

Variability in the bovine D-loop is concentrated in specific regions and a wider sequencing survey of a selected shorter sequence in 70 samples from four African and six European breeds has yielded further insight into the ancestry within the *Bos taurus* subspecies (Bradley *et al.* 1996).

In this, haplotypes from Africa and Europe clustered separately in phylogenetic analysis. All sequences from European breeds emerged in a star-like tree from a single, highly represented sequence, and all those from Africa formed a similar pattern around a second predominant haplotype that was separated from the European consensus by three substitutions. This pattern was

interpreted as evidence for the expansion of the two continental populations from two separate and possibly pre-domestic, ancestral sources. These may have been located in Anatolia (leading to European breeds) and the Eastern Sahara (resulting in modern African breeds) (Grigson 1989; Wendorf & Schild 1994). The earliest African cattle in nature are thought to have been *Bos taurus*, but most modern breeds are of zebu morphology (Epstein 1971). Despite this obvious and substantial genetic introgression of *Bos indicus* into the continent, all of the surveyed cattle displayed taurine mtDNA types. This is a striking illustration of the inertia of the primeval *Bos taurus* mtDNA gene pool in the face of major admixture (MacHugh *et al.* 1997).

The indigenous cattle of the Iberian peninsula are presumed to share a common Near-Eastern origin with those of the rest of Europe, although it has been speculated that local domestication of wild oxen may have contributed to the gene pool (Davidson 1989). Additionally, it has been suggested that the initial introduction of domestic animals to the peninsula occurred via a littoral Mediterranean route which may have included the North African coast (Payne & Hodges 1997; Waterbolk 1968). The peninsula, among European regions, has a uniquely intimate geographical and historical association with the African continent. Indeed, some authors refer to shared ancestry between Iberian cattle and breeds found in North Africa, although, to our knowledge, no genetic evidence has emerged to support such assertions (Feliú 1995). In this study we have assayed the mtDNA sequence in samples from six native Portuguese cattle breeds, chosen from both the temperate north of the country and the more arid, warmer South. The patterns of genetic variation revealed do not support a local aurochs contribution to Iberian cattle but do yield the first clear evidence for substantial introduction of African cattle into Europe.

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Figure 1. A reduced median network (Bandelt *et al.* 1995) featuring mtDNA lineages sampled in 49 cattle from Portugal. Circles represent sequence haplotypes; the area is proportional to the frequency of the haplotype. Shading indicates geographical origin. The two most frequent haplotypes are labelled A1 and E1 and represent the previously described African and European consensus sequences, respectively (Bailey *et al.* 1997; Bradley *et al.* 1996). The black dots denote theoretical intermediate nodes introduced by the median algorithm. Branches between the haplotypes represent substitutions; they are labelled by position as per Anderson *et al.* (1982), less 16 000. The single transversion is specified. Recurrent mutations are indicated by a and b, designating putative distinct mutation events at the same position. As with previous work involving European and African samples, the network shows two starburst phylogenies, in each of which a central, more frequent haplotype has given rise to a number of derivative variants. The recurrence of this pattern is a convincing demonstration of the presence of a mixture of typically European and typically African sequence families in the Portuguese population.

2. MATERIALS AND METHODS

(a) Sample collection

Blood samples were collected from six Portuguese breeds, Arouquesa (12), Alentejana (12), Barrosã (5), Maronesa (3), Mertolenga (12) and Preta (5). Arouquesa, Barrosã and Maronesa were sampled at the Centre of Animal Production in São Torcato (figure 1). Mertolenga were sampled at the Herdade da Abobada, Serpa. Preta were sampled in Porto Alto, Vila Franca de Xira and Alentejana in Portalegre. In all cases, the animals sampled came from pure-bred individuals, and pedigree information was used to ensure that they were not closely related. Whole DNA was purified from fresh blood using protocols previously described (MacHugh *et al.* 1997).

(b) DNA sequencing

Partial mitochondrial D-loops were amplified using the polymerase chain reaction. Primers were designed to amplify a 375 bp fragment from the most variable region AN4-bio (L15960; 5'-GGTAATGTACATAACATTAATG-3') and AN3 (H16334; 5'-CGAGATGTCCTTATTTAAGAGG-3'). Primer AN4-bio was biotinylated for subsequent product purification. Reactions were

performed using *ca.* 10 ng of template mtDNA in a 50 µl reaction volume, 2.5 units of *Taq* DNA polymerase, 10X reaction buffer (50 mM KCl; 10 mM Tris-HCl pH 9.0; 1% Triton X-100, 1.5 mM MgCl₂), with concentrations of 200 µM for each dNTP and 2 ng µl⁻¹ of both the forward and reverse primers. A 20 µl oil overlay was added to each sample before amplification. Amplification was performed in a Perkin Elmer Cetus thermal cycler using a 4 min denaturation step followed by 40 cycles of 40 s at 94 °C, 40 s at 55 °C, 40 s at 72 °C and a final extension at 72 °C for 4 min. Reaction products were purified using Dynabeads (DYNAL), according to the manufacturer's instructions. Sequencing of a 240 bp fragment (16 023–16 262) was performed using the dideoxy chain termination method with T7 polymerase (Pharmacia Biotech) and the AN3 primer.

(c) Sequence analysis

The 240 bp sequences were aligned by eye. A reduced median network was constructed using the methodology outlined by Bandelt *et al.* (1995). Pairwise F_{ST} distances were constructed according to Slatkin (1995) using the Arlequin analysis package (Schneider *et al.* 1997). The inter-population network was constructed from the resulting matrix using the

neighbour-joining algorithm (Saitou & Nei 1987) incorporated in the PHYLIP package (Felsenstein 1993).

3. RESULTS

(a) Variation in the bovine D-loop

In this survey, the most variable 240 bp region of the bovine D-loop was sequenced in 49 individuals representing six Portuguese cattle breeds. A comparison of the sequences revealed 26 haplotypes, which were differentiated at 32 polymorphic sites. These comprised 30 transitions, one single bp deletion and one transversion. Such patterns of variability are comparable with those described previously, particularly in their heavy bias towards transition substitution (Loftus *et al.* 1994; Bradley *et al.* 1996).

The sequence variants defining each haplotype are given in table 1. Note the sample of 26 unique sequences included both the European and the African consensus haplotypes identified in Bradley *et al.* (1996), here denoted as E1 and A1, respectively.

In total, samples matching the predominant European sequence were encountered 13 times and six animals possessed the African central haplotype. Four other variants were detected that shared the three defining African substitutions, one in two individuals and the others in single samples. The remaining sequences were of typical European type and of these, one haplotype was shared by three animals, three between two animals and 16 were detected only once. In addition to the two consensus sequences, only three haplotypes (P1, P2 and P3) matched sequences that had been described previously; those of three European samples (Bradley *et al.* 1996).

(b) Phylogenetic reconstruction

Figure 1 places each of the 49 sequences encountered in a network phylogeny constructed according to the rules outlined by Bandelt *et al.* (1995). In this, all haplotypes fall into two distinct clusters, each featuring a highly represented, centrally placed variant through which other sequences root to the rest of the phylogeny. Given the identity of the two central haplotypes with the previously described continental consensus sequences, it is clear that these two clusters are directly analogous to the African and European groups described by Bradley *et al.* (1996). The shading differentiates haplotypes encountered in southern and northern breeds and it is clear that the majority of the African haplotypes are found in the south. Notably, as before, most sequences are identical to, or fall within one or two bp substitutions of the presumed ancestral haplotypes. Using a substitution rate of 62.8% per million years, it may be calculated that a 240 bp fragment would display, on average, one bp change each 4300 years (Bradley *et al.* 1996). Therefore, the pattern of variation here is one that is roughly consistent with the derivation of almost all variants from one or other consensus sequence within domestic history. Haplotypes P9, P13 and P20 differ in three sites from the European nexus, E1, as does P22 from A1. The most divergent variant is P21, which differs from A1 by five substitutions. These variants possibly represent separate aurochs lineages, either domesticated locally or imported from the continental

centres of origin. However, ancient DNA analysis of wild oxen suggests that indigenous aurochs lineages might display more markedly divergent sequences (Bailey *et al.* 1996).

Pairwise F_{ST} values calculated using mtDNA data may be used to estimate genetic distances between populations over shallow time depths (Slatkin 1995). These were calculated here using the Portuguese breed data and an additional 69 published bovine D-loop sequences sampled from six European breeds (Friesian, Charolais, Simmental, Hereford, Jersey and Aberdeen Angus) and four African breeds (Butana, White Fulani, N'Dama and Kenana) (Bradley *et al.* 1996). These were used to construct a population-based, unrooted neighbour-joining phylogeny, which is shown in figure 2. The primary feature of this tree is the major branch separating the four African breeds from the European data set. Interestingly, the three southern Portuguese breeds (Mertolenga, Preta and Alentejana) have truncated branch lengths and are placed directly on this major branch. Their intermediate phylogenetic positions are a strong indication of African admixture. The three northern Portuguese breeds (Maronesa, Barrosã and Arouquesa) are positioned centrally within the European cluster.

(c) Admixture proportions

For each breed the proportion of mtDNA samples that displayed the three substitutions (at positions 16 050, 16 113, 16 255), which together are diagnostic of African haplotypes, were calculated. These are illustrated in figure 3, which also displays the approximate geographical origin of each variety. A cline in frequency is evident. The two breeds from the most northern areas, Barrosã and Maronesa, are devoid of detectable admixture. Whereas, the highest number of African haplotypes were found in the most southerly located breed, the Alentejana. In all, nine out of 29 samples taken from the south were of African mtDNA type, in contrast to only two out of 20 samples from the northerly area.

4. DISCUSSION

The cattle of Africa and Europe differ in a number of significant traits. Particularly, indigenous African breeds often possess valuable adaptations to local challenges such as heat tolerance and disease resistance (Murray *et al.* 1982; Hoste *et al.* 1988). Ancestral differences are also reflected by variation at the molecular level. Mitochondrial DNA sequences in each are of *Bos taurus* type but have been shown in phylogeny reconstruction to fall into two separate continental clusters separated by three substitutions. Analysis of genetic variation using a random selection of microsatellite markers has also illustrated the substantial divergence between taurine breeds of either continent, as well as quantifying the extensive introgression of *Bos indicus* autosomal genetics into all but a few relict West African populations (MacHugh *et al.* 1997). This paper, using the high resolution offered by mtDNA sequence analysis in six Portuguese cattle breeds, to our knowledge presents the first clear genetic evidence of partial African ancestry in a European cattle population.

Table 1. *Mitochondrial control region sequence variations observed in 49 cattle samples from Portugal*

(Sequence codes are given in the first column and the European and African consensus sequences are denoted A1 and E1, respectively. Only variable sites are shown. The sequence positions from the BOVMT GenBank sequence are given above each column (Anderson *et al.* 1982). Differences in E1 are given and a full stop (.) denotes the identity.)

[illegible]

Figure 2. An unrooted neighbour-joining tree summarizing pairwise F_{ST} genetic distances among six Portuguese, six other European and four African breed samples. Notably, northern Portuguese breeds (unfilled squares) cluster within the European clade (unfilled circles) and the breeds from southern Portugal (hatched squares) are situated at intermediate positions between the European and African groupings. The scale represents units of genetic distance derived from the original comparison matrix.

Figure 3. The proportion of African mtDNA haplotypes in each sample is illustrated by the filled sectors in the pie charts. The positioning of these symbols indicates approximate breed origins.

Iberian cattle are believed to share a common ancestry with other European cattle breeds, which traces back to the early domestic centres of the Near East (Grigson 1989). There has also been speculation that the wild oxen populations of the peninsula may have been domesticated locally (Davidson 1989). The phylogenetic analyses presented here do not fully support either of these assertions. First, it is clear that African as well as European cattle ancestry is represented in Portuguese cattle. The contribution of the former to the total mtDNA pool assayed here is 22% and this rises considerably to 42% in the Alentejana, the southernmost breed (figure 3). Second, mtDNA lineages derived from local aurochs, would be expected to feature as highly divergent haplotypes, as illustrated by ancient DNA analysis of British wild oxen (Bailey *et al.* 1996). Here, sequences gathered were typical of either previously described European or African haplotypes in their patterns of diversity (figure 1). Bradley *et al.* (1996) have argued that most, and perhaps all, lineages in either continental population derive from a respective highly represented consensus sequence that is central in phylogenetic topologies. In the data presented here, all European haplotypes were closely clustered around the predominant European haplotype, El, a pattern consistent with their derivation from imported domestic lineages. Only one African-type mtDNA chromosome, which exhibited five mismatches with the African consensus, provided a reasonable candidate for a relict African aurochs lineage. Although no divergent European haplotypes were encountered in this sample of 49 sequences, this does not preclude limited adoption of local animals, and particularly a contribution from wild bulls in the origins of Iberian cattle.

Possible explanations for the presence of African mitotypes in Portuguese cattle fall into three categories. First, the foundation herds of domestic cattle in Iberia may have been wholly or partially North African in origin. As the interior of the European continent consisted of largely impenetrable forest and highland, it is thought that the expansion of farming and livestock into the continent may have proceeded along the Mediterranean coast at a faster rate than through the continental heartland (Lewthwaite 1986; Waterbolk 1968). This route could have progressed along a corridor including the littoral of the Maghreb and may have involved the adoption of North African domesticates either at, or before, the Strait of Gibraltar, which represents a mere 13 km gap between the continents. Second, it is possible that historical conquests by North African imperial powers may have resulted in the inward migration of livestock. For example, the Carthaginians ruled the central and southern part of the Iberian peninsula until the second century BC, when it became part of the Roman empire. Also, and importantly, the Arabian Moors started an occupation in AD 711, which lasted for seven centuries and which heralded profound changes in architecture, language and culture, particularly in the southern areas of Spain and Portugal. Third, there is the possibility that the activities of Portugal as a later colonist of parts of Africa (e.g. Guinea Bissau, which possesses native herds of disease and heat-resistant N'Dama cattle (Hoste *et al.* 1988)), may have resulted in the deliberate importation of African livestock which possessed desirable breeding qualities.

Bovine mtDNA seems to be the most resistant part of the genome to introgression. For example, Africa and Anatolia represent two separate hybrid zones between introgressing *Bos indicus* and indigenous *Bos taurus*. In each case, substantial zebu autosomal admixture is not matched by zebu mtDNA influx (MacHugh *et al.* 1997; Loftus *et al.* 1999). This is easily explained as a consequence of primarily male-driven gene flow. Given these examples, it seems likely that the substantial African mtDNA presence in Portugal is indicative of an extensive influx of cattle from the southern continent. Also, there is a cline in the frequency of African variants, with a stronger influence discernible in the southern breeds. These facets of the data argue against a later, Portuguese colonial origin for the introgression, which would probably have been localized, of limited extent and which would not necessarily have had a greater input to southern cattle.

Both of the remaining hypotheses are consistent with a substantial and widespread admixture. However, a primeval hybridization following an initial dispersal of domestic cattle from the Near East through a Mediterranean littoral corridor is somewhat more difficult to reconcile with the geographically gradated nature of the variation. One would expect an initial admixture with North African cattle en route to have spread evenly throughout the peninsula, whereas here, African haplotype frequencies and phylogenetic analysis both indicate a difference between northern and southern breeds. However, a north-south disparity could perhaps have resulted from later European influx and an African component in the first Iberian cattle cannot be entirely discounted.

The more likely explanation for the observed introgression lies with the migratory influxes of later, North African conquerors. It has been acknowledged that the Moors, in particular, have had a major influence on the peninsula. Lisbon, for example, initially fell to the Arab conquerors in the eighth century and remained under Muslim control until 1147. They are known to have introduced new crops, such as sugar cane, rice and cotton, and also Merino sheep. The cline in frequency here could possibly reflect the differences in climate between the north and south, and the boundary of increased use of African animals, which would have possessed inherent genetically controlled adaptations to heat and other warm-climate challenges. Additionally, the north of the peninsula was only briefly occupied by the Moors during the height of their influence; in sharp contrast to their prolonged and intimate association with the south.

The modern cattle of most of Africa possess a substantial degree of *Bos indicus* autosomal genetics (Bradley *et al.* 1994; MacHugh *et al.* 1997). However, it is thought that they only moved to West Africa in significant numbers with the Arab invasions after the death of the Prophet in AD 670. (Epstein & Mason 1984). Published genetic polymorphism data in Iberian breeds suggests some zebu ancestry in addition to European and African influences. For example, the serum albumin B allele is nearly fixed in pure *Bos indicus* breeds and almost absent in unintrogresed *Bos taurus* breeds (Baker & Manwell 1980). Notably, it is found at appreciable frequencies in some breeds in the peninsula; for example, at 0.16 in Mertolenga and 0.44 in the Spanish Blanca Cacerena (Kidd *et al.* 1980; Gonzalez *et al.* 1987), which suggests admixture within the last 1300 years. The Moorish empire was part of the same remarkable cultural and military expansion that spread *Bos indicus* genetics throughout most of North and West Africa, and it seems likely that its influence is also reflected in the modern genetics of Portuguese cattle.

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