

## Genetic signatures of a Mediterranean influence in Iberian Peninsula sheep husbandry

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Keywords: *Ovis aries*, mtDNA lineages, Iberian Peninsula, sheep husbandry

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Running title: Sheep husbandry in SW Europe

## **Abstract**

Highly adaptable and versatile populations of domestic sheep, the result of millennia of intense husbandry, are found in almost every corner of the world. Here we describe a genetic survey of sheep from the western fringe of its European distribution. We studied the mitochondrial DNA control region sequences from 161 individuals belonging to seven Portuguese sheep breeds. Our study revealed a high level of genetic diversity, with an average breed haplotype diversity of 0.983, substantially above that observed in central European breeds, as well as the presence of maternal lineages until now only found in the Middle East and Asia. A broad North-South pattern describes the most important trend in the Portuguese sheep population with a southern population clearly distinct from most other breeds. A recurrent influx of new genetic diversity, probably via the Mediterranean Sea, may explain these patterns and appears to corroborate the importance of this maritime route in the history of both mankind and livestock. Zoo-archaeological studies of sheep bones from southern Portugal indicate a marked size increase during the Moslem period which may reflect an improvement of this animal – perhaps part of the well known ‘Arab Agricultural Revolution’ in Andalusia. This could have been a time when the gene pool of Iberian sheep was substantially enriched and may help to explain the history of modern sheep breeds in this Peninsula.

## Introduction

The beginning of livestock domestication, some ten to eleven thousand years ago in the 'Fertile Crescent', was a crucial event in human history. The earliest zoo-archaeological evidence for domestic sheep comes from sites in present day Iran, Turkey and Cyprus (Hahn 1896; Uerpmann 1979; Ryder 1984; Clutton-Brock 1999, Vigne et al. 2003). A pioneering genetic study which examined the karyotypes of the various species of extant wild sheep (Nadler et al. 1973; Bunch et al. 1976) clearly showed that our domestic sheep derive from the Asiatic mouflon (*O. orientalis*) of Anatolia, western and South-West Iran rather than any of the other species of wild Asiatic sheep like the Urial (*O. vignei*) and the Argali (*O. ammon*). Recent genetic techniques, using mitochondrial DNA (mtDNA) sequence variation, have further refined these early findings by supporting a South-West Asiatic origin for domestic sheep and excluding any contributions from the Urial and the Argali (Hiendleder et al. 1998b; 2000).

Phylogenetic analysis of domestic sheep mtDNA has revealed the existence of two main haplogroups (HGs), termed A and B. They were first identified in populations of New Zealand sheep (Wood and Phua 1996) and subsequently confirmed in Eurasian breeds. HG B is widespread while HG A appears to be almost entirely confined to Asiatic animals (Hiendleder et al. 1998a; 1998b; 2000). Recently, a third HG (C) was identified in Middle Eastern (Pedrosa et al. 2005; Bruford and Townsend 2006) and Asiatic sheep (Guo et al. 2005) which strongly suggests multiple domestication events similar to the pattern identified in other domestic species such as goat (Luikart et al. 2001) cattle (Troy et al. 2001) and pig (Larson et al. 2005).

Archaeological evidence indicates a probable spread of the Neolithic "package" out of the Near East and across Europe. The routes taken could have been either through the continental heartland up the Danube valley or along the Mediterranean coast (Ryder 1984). The first appearance of the remains of domestic sheep in the western part of Mediterranean Europe date to approximately 5,400 BC and are believed to reflect a rapid spread by sea - part of a process of maritime pioneer colonization (Zilhão 2001). To what extent the Mediterranean route was used in the following millennia for the transport of domesticated animals is however unclear. In fact, the influence of different post-Neolithic cultures in southwest Europe is now becoming increasingly evident (see Table 1, Supplementary Material, and references cited therein). Many of these colonists who came to the Iberian Peninsula like Phoenicians, Greeks, Romans and Berbers, probably introduced new species of animals and new breeds of

livestock, and most of them came by sea. Some colonists may have improved local livestock as well as importing stock from overseas.

The oldest established sheep breed in the world, the Merino, was developed in Iberia. Klein (1920; 3-6) suggested that this breed was introduced into the Iberian Peninsula by, and named after, the Beni Merin Berbers, one of the North African tribes that settled in large numbers in the southern part of Iberia while under Almohad rule (from the mid 12<sup>th</sup> century). Moslems introduced new techniques – especially those concerning irrigation - that enabled the growth of exotic varieties like sugar cane, rice, cotton, and citrus trees. This is referred to as the ‘Arab Agricultural Revolution’ (Watson 1974; Glick, 1979; El Faiz 2000). But while the literature speaks much of oranges and lemons, and apart from the famous Arab horses, we know little about the rest of the livestock sector.

Patterns of genetic diversity found in modern sheep breeds from Iberia that could shed some light upon the history of circum-Mediterranean migrations are largely unknown. Therefore, we attempted to characterize the female side of sheep history by studying the mtDNA of seven Portuguese breeds, belonging to the three main Iberian branches: Merino, Churra and Bordaleiro.

Our results show an extremely high level of genetic diversity and the presence of lineages until now only found in the Near East and Asia. The same pattern was found in domestic goats (Pereira et al. 2005) providing molecular evidence for an intense gene flow between the Near East and Iberia via a Mediterranean - perhaps maritime - route.

The combination of our genetic data with archaeological evidence for the improvement of sheep during the Moslem period in Portugal (Davis 2005), strongly suggest that the Iberian Peninsula has been an important centre for development and improvement of this domesticated.

## Materials and Methods

### *Sample collection and DNA extraction*

We collected bloodstains on FTA cards (Whatman) from 161 unrelated sheep belonging to seven Portuguese breeds: Churra Badana (n=26), Churra da Terra Quente (n=24), Mondegueira (n=14) and Churra Algarvia (n=35) from the Churra Type; Campaniça (n=20) and Saloia (n=32) from the Bordaleiro Type and Merino Preto (n=10) from the Merino Type. All animals sampled belonged to pure indigenous breeds and were registered with their respective breeders association to ensure that they were not closely related. We performed DNA extraction according to the Chelex 100 method.

### *DNA amplification and sequencing*

The complete mtDNA control region between positions 15437-16616, according to the ovine reference sequence (Hiendleder *et al.* 1998b), was amplified in two overlapping fragments using two pairs of primers (for fragment 1: SIF: 5'-CCCCACTATCAACACCCAAA-3' and SIR: 5'-CATGGTGAACAAGCTCGTGA-3' and for fragment 2: SIIF: 5'-TGCTTGACCGTACATAGTACAT-3' and SIIR: 5'-CATCTAGGCATTTTCAGTGCC-3'). PCR products were sequenced on both forward and reverse directions on an ABI 3100 Automated Sequencer using the Big Dye Sequencing Kit (AB Applied Biosystems).

### *Data analysis*

The sequences obtained were compared by alignment to the ovine reference sequence [AF010406; Hiendleder *et al.* 1998b)] and have been deposited in GenBank with the accession numbers: DQ491576-DQ491736. A large database was constructed using all complete mtDNA control region sequences available in GenBank: L29055 (Zardoya *et al.* 1995); Z35228-Z35268, Z35293 (Wood and Phua 1996); AF039577-AF039578 (Hiendleder *et al.* 1998b); AF010406-AF010407 (Hiendleder *et al.* 1998a); AY091495-AY091500 (Hiendleder *et al.* 2002); AY829376-AY829430 (Guo *et al.* 2005); AY582800-AY582820 (De Ascencao *et al.* 2004); as well as all available partial sequences - DQ097431-DQ097468 (Pedrosa *et al.* 2005) and AY879343-AY879463 (Meadows *et al.* 2005).

Diversity measures, AMOVA,  $F_{ST}$  genetic distances, exact test of population differentiation, mismatch distributions and the Fu's  $F_s$  statistic were all obtained using *Arlequin 2.0* (Schneider *et al.* 200). The  $F_{ST}$  genetic distance matrix was summarised in two major trends or dimensions using multidimensional scaling (MDS) analysis as implemented by the *STATISTICA* program (StatSoft, Inc. 1999). The value for each breed in each dimension can then be used to interpolate a synthetic surface map, which illustrates geographically the major genetic trends across the Portuguese sheep population. A geographic coordinate for each breed's place of origin, was taken as a central point in the relevant sampling area. Maps were constructed using the *Spatial Analyst v2.0* extension of *ArcView GIS 3.2*. Median-joining networks (Bandelt *et al.* 1999) were calculated using *Network 4.1.1.0* ([www.fluxus-engineering.com](http://www.fluxus-engineering.com)) with positions weighted in inverse proportion to the maximum number of mutations obtained for each position in a first run.

## Results

### *MtDNA variation in Portuguese sheep breeds*

The complete mtDNA control region (~1180 bp) sequences from 161 unrelated animals revealed 134 different haplotypes with 195 polymorphic sites. Four copies of a tandem repeated motif in the tRNA<sup>Pro</sup> proximal part of the control region were found in all individuals with the exception of two sheep, from the Saloia and Badana breeds, which each had three copies.

Most individuals (93.8%) belong to HG B, seven to HG A (4.3%; from Saloia, Churra Badana, Churra da Terra Quente and Churra Algarvia breeds) and three to HG C (1.9%; from Churra Badana and Churra Algarvia breeds). Only two haplotypes were found to be shared between breeds (Saloia and Churra Algarvia). A high diversity was observed in all breeds with average haplotype diversity of 0.985 and mean number of pairwise differences ranged between 10.621 and 12.359 (Table 2, Supplementary Material). In order to discern the affinities between different Portuguese breeds, pairwise  $F_{ST}$  genetic distances were calculated and an exact test of population differentiation performed. They show that the southern breed (Churra Algarvia) presents the largest genetic distances when compared with other breeds (Table 1). Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) revealed that most of this variation (98.5%) occurs within breeds, while just 1.5% is due to the variation between breeds.

MDS analysis of the matrix of interbreed  $F_{ST}$  values was performed and the geographical variation in each dimension was visualized by interpolating observed values to produce a synthetic surface map of Portugal. A broad North–South trend was observed for the first dimension, with the extreme southern breed (Churra Algarvia) standing out as the most differentiated (Figure 1). The correlation between dimension 1 values and the latitude of the sample populations is positive ( $R^2=0.48$ ,  $p=0.084$ ). No discernable trend was observed for the second dimension (data not shown).

### *Ovine mtDNA lineages phylogeography*

All publicly available mtDNA control regions sequences (n=449) were assembled into a single database. Alignment of these sequences covering nucleotide positions 16093-16616 only, to allow maximum comparability between published data, defined 193 different

haplotypes. The phylogenetic relationship between these was reconstructed in a median-joining network (Figure 1, Supplementary Material). The Portuguese sequences fall into the three previously identified HGs (A, B and C), which are readily apparent in the network.

HG B is the most widespread, reaching a frequency of 75% followed by HG A (19%) and HG C (6%). A median-joining network calculated for HG B haplotypes revealed a complex distribution of lineages, with Portuguese sequences scattered along different branches (most European haplotypes identified in the network B of Figure 2). The most frequent haplotype is central to the network and was found worldwide, including several Iberian sheep. Three haplotypes shared by Portuguese and Middle Eastern breeds and one by Portuguese and Asiatic animals were found just one mutational step away from this central haplotype.

The distribution of Hg B lineages, calculated for breeds with at least 10 sampled individuals, revealed that all Portuguese breeds present a substantially higher haplotype diversity than the central European ones. Even when compared with a mixed sample composed of individuals from five different Turkish breeds, the Portuguese populations presented a higher diversity of maternal lineages (Table 2).

The median-joining network calculated for HG A haplotypes revealed a simpler pattern in which most sequences are just one mutation derived from the most frequent haplotype – the probable ancestor of this HG (network A of Figure 2). Although extremely rare in Europe, we found considerably different lineages in the Portuguese sample: two from the central haplotype, two one step derived and three in distant branches of the network.

We also identified, for the first time, European haplotypes belonging to HG C. Remarkably, two sequences previously assigned to HG C (Guo et al. 2005) were connected, although distantly, to HG A in the network constructed with all sequences (Figure 1, Supplementary Material). A careful inspection of these sequences revealed that they present several unique polymorphic positions. The three Portuguese sequences belong to two different lineages on distinct branches of the median-joining network of HG C (network C of Figure 2). The central haplotype is shared by Asiatic and Near Eastern breeds, but the scarce number of sequences available so far prevents us from drawing more definite phylogeographic conclusions.

## Discussion

Both archaeological and genetic evidence indicate that the domestication of wild sheep occurred within a somewhat restricted area of South-western Asia some ten or eleven thousand years ago. In the following millennia domesticated sheep were spread rapidly into different regions – a spread which is generally associated with the expansion of Neolithic communities (Ryder 1984). Subsequent selective breeding for desirable traits such as wool, milk and meat production as well as environmental tolerance, was responsible for the development of highly productive and well adapted sheep populations. The original location of the first appearance of the most important improvements in animal breeding is still a matter of debate.

Here, we present genetic data consistent with a continual Mediterranean influence upon Iberian sheep populations. Although on the periphery of the distribution of Eurasian sheep, the seven Portuguese breeds studied by us reveal a remarkably high level of genetic diversity in their mitochondrial gene pool when compared with other European breeds (Table 2). In the absence of a continuous gene flow, we might expect a decrease in diversity as the distance increases from the hearth of domestication. This pattern is observed in cattle (Troy et al. 2001) and, according to some, also in sheep (Bruford and Townsend 2006). Interestingly, our data do not support these claims, and reveal instead a high degree of haplotype and HG diversity in Iberia, similar to the one previously detected in goats (Pereira et al. 2005). Considerable genetic variation has also been revealed by autosomal microsatellite markers in several Iberian breeds, particularly in the Merino type (Arranz et al. 1998; Diez-Tascón et al. 2000; Rendo et al. 2004).

Although the differentiated geographical distribution of the three ovine HGs suggests that HG B had witnessed a much older population expansion than HG A or HG C, both the occurrence of HG C in our sample and the pattern of diversity observed within each of them do not support this scenario. Sheep domestication may have occurred within a relative short time, when compared to what has been implied in goats (Luikart et al. 2001). Indirect evidence supporting this hypothesis can be seen in the mismatch distribution graphs and also from the mean number of pairwise differences which are not significantly different among the three HGs (Figure 3; Supplementary Material).

Phylogenetic examination reveals that the Portuguese lineages fit within the three main sheep HGs previously described. The unique observation of HG C in our study is the first evidence for the presence of this infrequent HG outside the putative domestication areas. Moreover, HG A, previously referred to as an “Asiatic lineage” (Hiendleder et al. 1998b) due

to its high frequency in breeds of Asiatic origin, was also identified in four Portuguese breeds with considerably divergent lineages (network A of Figure 2). It is most interesting that when compared to a previous study, in which almost 700 individuals were analyzed (Bruford and Townsend 2006), there is a significant difference ( $p < 0.001$ ) between the proportion of sequences from HGs A and C in the Portuguese sample (9 out of 161) when compared with the proportion in the remainder of Europe, where less than 10 HG A lineages were identified.

The presence of these rare lineages and the high diversity found in Iberia can be explained either as the legacy of ancient introductions or the product of more recent introgression(s) from imported oriental breeds over the past 150 years of intense modern breeding. Our results tend to support the former hypothesis since: (i) we sampled only pure indigenous breeds from small villages avoiding recently improved animals from intensive, industrial breeders; (ii) there is no historical reference to recent crossing of Portuguese breeds with animals from the Near East or Asia; (iii) there are considerable morphological differences between Iberian breeds (thin-tailed sheep) and Near Eastern and most Asiatic breeds (fat-tailed and fat-rumped breeds) and (iv) the Portuguese representatives of the “Asiatic” HG show a profound sequence heterogeneity and are scattered in the corresponding network.

In this respect another related and important question is the route or routes used for the introduction of domestic sheep into Iberia. The most plausible explanation for our results is the extensive use of a Mediterranean route with the transport of animals made by sea and/or along Mediterranean coastal regions. Indeed if a central European continental pathway had been the main route, it would have left its imprint along the way, with a higher frequency of these HGs in Europe. Since we still know very little about the nature of the genetic pattern of North African sheep, the importance of a terrestrial route along the African Mediterranean coast and its influence upon Iberia, either during or before the Moslem period, remains to be clarified. However, evidence from the Cairo Genizeh indicates quite clearly that in the 11th and 12th centuries the Mediterranean world was a kind of medieval common market forming a free trade area (Goitein 1967). This communications network, shared by Christians, Jews and Moslems expressed the notion that there was blessing in movement “fi’l-haraka baraka” (Glick 1979; 27). So too the Atlantic maritime trade between Spain, Portugal and the Maghreb at this time is well documented by Picard (1997).

Another signature of a direct Mediterranean influence in Iberia is the North–South trend observed for the interbreed genetic distances (Figure 1). The influx of new stock through the south of the Iberian Peninsula is compatible with this trend, as well as with the fact that the southernmost breed (Churra Algarvia) is the one that displays the highest degree of

differentiation and the greatest interbreed genetic distances. This interpretation does not result from the presence of “exotic” A or C HGs, which are relatively rare and shared with other breeds, but mainly from the peculiar patterns of sequence observed inside HG B, as they are located at the tips of long branches of the Portuguese HG B network (Figure 2; Supplementary Material). Previous studies, revealing an unexpected high diversity in breeds of Iberian domestic goats (Pereira et al. 2005), and a differential cattle migration along the Mediterranean coast (Cymbron et al. 2005) further substantiate the importance of the Mediterranean Sea in the history of the movement of people and their livestock.

Both archaeological and historical evidence indicate that after the Neolithic the Iberian Peninsula experienced extensive contact with the outside world, leading to the introduction of new crops and animals (Table1, Supplementary Material). Besides prehistoric contacts, a possible influx of domestic sheep could have occurred when the Phoenicians and Greeks established their important trading colonies in the southern part of Iberia. These lasted several centuries. Important developments in farming methods also occurred during Roman times with probable consequences for sheep husbandry. However, the clearest evidence for an improvement of Iberian sheep is to be seen in the Moslem period. A substantial osteometric change in this period is identified in sheep bones from southern Portugal collected from a succession of archaeological sites dating from the third millennium BC to the present day (Davis 2005). This observation suggests a significant improvement of local stocks during the Moslem period, which, as is well known, had a major and long-lasting influence on the economy and culture of Iberia between the early 8<sup>th</sup> to the late 15<sup>th</sup> century.

Both genetic and zoo-archaeological observations on modern and ancient Portuguese sheep highlight the important role that Iberia played in livestock developments. Despite its position on the western edge of continental Europe, the Mediterranean Sea acted as a natural corridor connecting the peninsula to the Near East, North Africa and Southern Europe either through long distance migratory movements or shorter ones, like the crossing of the Straits of Gibraltar. This, together with climatic similarities to the Near East, may have allowed the influx of genetic diversity essential for selective breeding of desirable traits. This is particularly plausible for domestic sheep and goat given their environmental adaptability and the ease with which they can be transported (see Luikart et al. 2001).

Particularly relevant in Iberia must have been the continued arrival of new civilizations that brought with them new agricultural techniques. These may well have included selective breeding of smaller ruminants such as sheep. One other factor that is worth bearing in mind is the enormous topographic and climatic diversity found within Iberia. This peninsula

encompasses extremely arid regions, a sub-tropical Mediterranean climate and mountainous areas with extremely cold winters; a climatic and environmental diversity that may well have stimulated the development of different specialized breeds.

The importance of Iberian sheep husbandry is evident in the successful types of sheep that are found in the region today. The most remarkable of these is the fine-wool Merino which was probably developed during the first Greek incursions into Iberia, more intensely during the later Islamic period, and subsequently with the establishment by 'Alfonso the Learned' in 1273 of the Mesta – the sheep holders association in Castile (Klein, 1920). From the 18<sup>th</sup> century onwards Merinos were spread all over the world and are now the most numerous sheep breed and the major source of the world's wool supply. Crosses between Merino and other sheep breeds have given rise to several of the world's most productive breeds, such as the Ile-de-France and Merino-Landschaf. Another Iberian sheep breed, the carpet-wool Churra, was very important in the colonization of the New World and was the first to be introduced into the Americas (Ryder 1983; 1984).

Finally, the amount of genetic diversity found in Iberian breeds of sheep also shows that management and conservation strategies must involve regions located further away from the putative centers of domestication.

## **Acknowledgements**

We are grateful to Barbara van Asch for critical comments and helpful discussions and Arturo Morales Muñoz for kindly checking and correcting the table of early animal introductions. We are also grateful to Casimiro Escudeiro (Associação Nacional de Criadores de Ovinos da Raça Churra da Terra Quente), Pedro Ivo (Associação de Produtores de Pequenos Ruminantes da Bacia Hidrográfica do Côa), Rita Guerreiro and Vânia Azinheira (Associação de Criadores de Gado do Algarve), Miguel Madeira (Associação de Criadores de Ovinos do Sul), Dina Martins (Associação de Criadores e Reprodutores de Gado do Oeste) and to the Associação Nacional de Criadores de Ovinos da Raça Churra Badana for the collection of blood samples, and SD is, as always, grateful to John Watson for useful discussions. This work was partially supported by a research grant to FP (SFRH/BD/19585/2004) from Fundação para a Ciência e a Tecnologia and IPATIMUP by Programa Operacional Ciência, Tecnologia e Inovação (POCTI), Quadro Comunitário de Apoio III.

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Table 1:  $F_{ST}$  genetic distances between the Portuguese sheep breeds (below diagonal) with  $P$ -values in parentheses. Significant ( $P$ -values) of exact tests of population differentiation are shown above the diagonal.

|                  | Churra Badana          | Churra T. Quente       | Mondegueira            | Churra Algarvia        | Campaniça              | Saloia                 | Merino Preto |
|------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|--------------|
| Churra Badana    | -                      | 0.030±0.012*           | 0.030±0.006*           | 0*                     | 0.016±0.008*           | 0.034±0.010*           | 0.065±0.011  |
| Churra T. Quente | 0.010<br>(0.027±0.002) | -                      | 0.139±0.016            | 0*                     | 0.120±0.026            | 0.203±0.019            | 0.299±0.029  |
| Mondegueira      | 0.019<br>(0.017±0.001) | 0.012<br>(0.021±0.002) | -                      | 0*                     | 0.058±0.014            | 0.189±0.018            | 0.123±0.009  |
| Churra Algarvia  | 0.023<br>(0)           | 0.016<br>(0.003±0.001) | 0.025<br>(0.011±0.001) | -                      | 0*                     | 0.001±0.000*           | 0*           |
| Campaniça        | 0.014<br>(0.011±0.001) | 0.007<br>(0.041±0.002) | 0.016<br>(0.028±0.002) | 0.020<br>(0.003±0.001) | -                      | 0.086±0.015            | 0.179±0.015  |
| Saloia           | 0.010<br>(0.004±0.001) | 0.004<br>(0.098±0.003) | 0.013<br>(0.012±0.001) | 0.015<br>(0.001±0.000) | 0.007<br>(0.026±0.002) | -                      | 0.398±0.031  |
| Merino Preto     | 0.019<br>(0.049±0.002) | 0.012<br>(0.096±0.003) | 0.022<br>(0.097±0.003) | 0.026<br>(0.037±0.002) | 0.016<br>(0.098±0.003) | 0.013<br>(0.072±0.003) | -            |

\* Significant values ( $p < 0.05$ )

Table 2: MtDNA haplotype diversity observed in sheep breeds, with at least 10 Haplogroup B individuals sampled, using a control region segment from positions 16093-16616.

| Breed                           | <i>n</i> | N° of Haplotypes | Haplotype Diversity |
|---------------------------------|----------|------------------|---------------------|
| Portuguese:                     |          |                  |                     |
| Churra Badana                   | 23       | 19               | 0.980 ± 0.020       |
| Churra Terra Quente             | 23       | 22               | 0.997 ± 0.014       |
| Mondegueira                     | 13       | 11               | 0.974 ± 0.039       |
| Churra Algarvia                 | 32       | 19               | 0.966 ± 0.014       |
| Campaniça                       | 20       | 18               | 0.990 ± 0.019       |
| Saloia                          | 29       | 28               | 0.998 ± 0.010       |
| Merino Preto                    | 10       | 9                | 0.978 ± 0.054       |
| Central European <sup>a</sup> : |          |                  |                     |
| Carynthian Sheep                | 18       | 6                | 0.680 ± 0.109       |
| Forest Sheep                    | 10       | 6                | 0.778 ± 0.138       |
| Tyrolean Stone Sheep            | 12       | 7                | 0.909 ± 0.056       |
| Tyrolean Mountain Sheep         | 12       | 8                | 0.924 ± 0.058       |
| Turkish <sup>b</sup>            | 19       | 12               | 0.953 ± 0.028       |

<sup>a</sup> Meadows et al. 2005

<sup>b</sup> for a mixed sample of individuals from five different Turkish breeds, Pedrosa et al. 2005

## Figure Legends

Figure 1: Synthetic map of Portugal illustrating the geographic variation in the first dimension of genetic diversity obtained from MDS analysis of interbreed  $F_{ST}$  values. Crosses indicate sample locations, as follows: Churra Badana (CB), Churra da Terra Quente (CT), Mondegueira (MO), Saloia (SA), Campaniça (CA), Merino Preto (MO) and Churra Algarvia (AL).

Figure 2: Median-joining networks of the mtDNA haplotypes, found in Haplogroup A, B and C, using a control region segment from positions 16093-16616. European or European derived (Mexican sequences) haplotypes are in black; Asiatic in white; Near and Middle Eastern in dark grey and New Zealand in light grey. For Haplogroups A and C sequences unique to Portugal are denoted by a 'P', while for Haplogroup B the Portuguese sequences are distributed throughout the network. The area of the circles is proportional to the frequency of specimens in the sample.

Figure 1

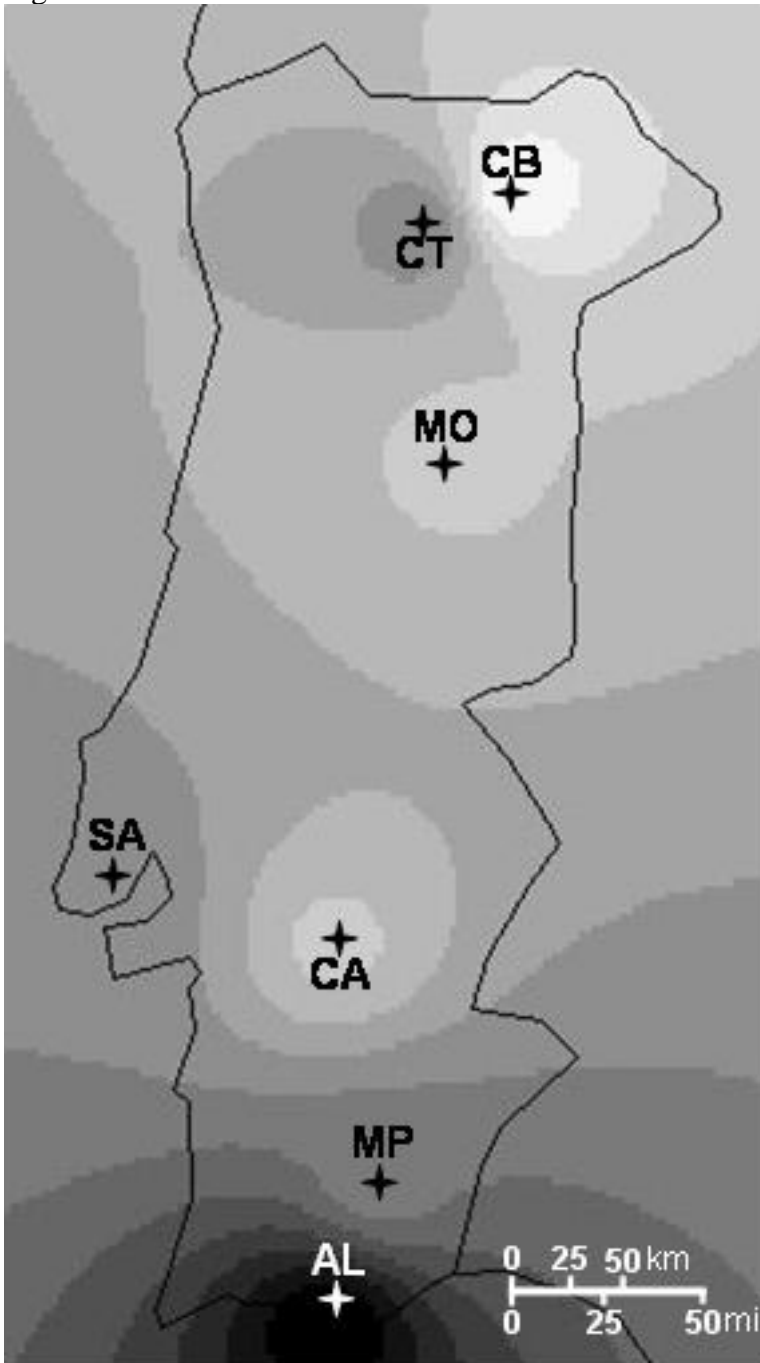


Figure 2

