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# Haplotypic diversity of porcine *LEP* and *LEPR* genes involved in growth and fatness regulation

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**Abstract** The analysis of structural genetic variability in candidate genes can make it possible to analyse the selection footprint and deepen the understanding of the genetic basis of complex traits. The leptin (*LEP*) and its receptor (*LEPR*) porcine genes are involved in food intake and energy homeostasis, and polymorphisms associated to growth and fatness traits have been detected in both genes. The main objective of this study was to explore the genetic variability of the most polymorphic regions of both genes in a variety of pig populations and wild boars from diverse European and Asian origins. In total, 54 animals were included in the analyses, with a remarkable sampling of Spanish wild boars and Iberian pigs. The sequencing allowed the identification of 69 and 26 polymorphisms in *LEP* and *LEPR* genes, respectively. Neighbour-joining trees built for the 69 haplotypes identified in the *LEP* and the 24 haplotypes detected in the *LEPR* showed the known genetic divergence between European and Asian pig breeds. A high variability of the *LEP* was detected in the different analysed populations providing new data for the existence of two domestication centres in Asia. In comparison to the *LEP* gene, the *LEPR* showed a lower variability, especially in the Iberian breed that showed no variability. Moreover, results of the Hudson-Kreitman-Aguadé neutrality test support a possible selection event of the *LEPR* gene region in this breed, potentially related with its leptin resistance pattern and good adaptation to a traditional extensive production system with strong seasonal changes of feeding resources.

**Keywords** Haplotype · Iberian pig · *LEP* · *LEPR* · mtDNA · Selection

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## Introduction

The genetic variability of livestock species has been shaped by diverse forces such as multiple domestication events, population admixture, natural selection and selective breeding. Since the beginning of the 1990s, molecular data have played an essential role in surveying the genetic variation within and between breeds of farm animals (Frankham et al. 2002). Most of the studies performed on pigs have been conducted using specific mitochondrial DNA (mtDNA) regions (Giuffra et al. 2000; Kim et al. 2002; Alves et al. 2003; Luetkemeier et al. 2010) or microsatellites markers. (Laval et al. 2000; Gama et al. 2013). Recently, a phylogenic analysis of complete genome sequences of wild boars and Asian and Western domestic pigs substantiates the hypothesis that pigs were independently domesticated in Eurasia and East Asia and supports the Asian influence in most of the cosmopolitan European and American breeds (Groenen et al. 2012). Moreover, the new massive parallel sequencing technologies have allowed for the identification of some candidate regions within the porcine genome that putatively have been under selection for diverse goals (Amaral et al. 2011; Wilkinson et al. 2013).

The analysis of the genetic variability in particular candidate genes related to quantitative traits can deepen the understanding of the genetic basis of such complex traits (D'Andrea et al. 2008; Yang et al. 2012). Because modern selective breeding towards leaner pigs must have dramatically affected

the regulation of biological pathways underlying growth and fatness, it will be particularly interesting to know the selection footprint left on genes related to these traits (Ojeda et al. 2006). The detection of selection requires the identification of departures from the expected patterns of molecular variation under neutral conditions when selection is absent. There have been previous studies aimed at analysing the genetic diversity of singular genes associated with growth and fatness traits such as *IGF2* (Ojeda et al. 2006), *FABP4* (Ojeda et al. 2008), *SERPINA6* (Esteve et al. 2011), *PPARD* (Ren et al. 2011), *FTO* (Fontanesi and Russo 2013) and *MUC4* (Yang et al. 2012). The objective of the present study was to analyse the variability in different pig populations of two key genes, the *leptin (LEP*) and its receptor (*LEPR*), implicated in the control of feed intake and energy balance and therefore in the regulation of body mass and composition. These genes have been widely studied in pigs due to their relevance on important economic traits (Galve et al. 2012; Wylie 2011; Switonski et al. 2010; Óvilo et al. 2002) and the *LEP* gene is considered a hot spot with an extensive amount of polymorphisms (D'Andrea et al. 2008). Our previous studies on an Iberian x Landrace experimental cross reported significant effects for pig productive traits of SNPs located in both genes (Ovilo et al. 2010; Pérez-Montarelo et al. 2012, 2013). The study of these genes is especially interesting in the extremely fat Iberian pigs, whose adipogenic phenotype corresponds to a *leptin resistance* pattern showing much higher voluntary feed intake and levels of circulating leptin than other leaner breeds (Morales et al. 2002; Fernández-Figares et al. 2007). Moreover, this breed carries a fixed *LEPR*c.1987 allele. The 1987 T allele, that reduces the hypothalamic *LEPR* expression and leptin protein signaling, enhances feed intake and increases fat accumulation and growth (Ovilo et al. 2010). Previous analysis of the *LEP* and *LEPR* gene sequences allowed us to identify their most polymorphic regions, which correspond to the promoter region of the *LEPR* gene (Pérez-Montarelo et al. 2013) and the intron between exons two and three of the *LEP* gene (Pérez-Montarelo et al. 2012). In the present study, the analyses of these *LEP* and *LEPR* polymorphic regions have been conducted in Iberian pigs and several other pig populations from different origins to explore their haplotypic diversity and to obtain further insight into the breed-specific role of both genes on the control of voluntary feed intake and related productive traits.

## Materials and methods

### Pig breeds and specimens

A total of 54 animals were included in the analyses (Table 1), comprised of 29 domestic pigs from nine different European and American (Western) breeds. The included samples were

**Table 1** Wild boars and domestic pigs analysed in this study: presence of European and Asian *Cytochrome B* haplotypes in the different groups based on the nomenclature employed by Fang and Andersson (2006) according to the polymorphism of six nucleotide positions (15,035, 15,036, 15,038, 15,041, 15,044 and 15,045)

| Population/ breed | *n* | *Cytochrome B* haplotype[§] |
|---|---|---|
| Iberian | 12 | E1 |
| Spanish wild boars | 9 | E1 |
| Romanian wild boars | 2 | E1 /E3 |
| Iranian wild boars | 2 | E2 |
| Duroc Jersey | 2 | E1/A1 |
| Duroc | 6 | E1/A3 |
| Landrace | 2 | E1 |
| Large Black | 1 | E1 |
| Ancient Large White | 2 | E1 |
| Large White | 2 | A1/A3 |
| Pietrain | 2 | A1/A2 |
| Youli | 2 | A1 |
| Siberian wild boar | 1 | A1 |
| Fengjing | 1 | A2 |
| Meishan | 2 | A1/A3 |
| Vietnamesse | 6 | A1/A2/A3 |

[§] European (E1: CACGTC, E2: CACATC, E3: CACGTT), Asian (A1: CGTATT, A2: CGTATC, A3: CATATC)

12 Iberian pigs from different varieties and strains (Torbiscal, Entrepelado, Retinto and Lampiño) representative of the breed (Fabuel *et al*. 2004), and 17 pigs from other Western breeds: American Duroc (*n*=6) and ancient Duroc Jersey (*n*=2), European Landrace (*n*=2), Large White (*n*=2), ancient Large White (*n*=2) preserved in Spain from 1931, Pietrain (*n*=2), and the endangered breed UK Large Black (*n*=1). In addition, 13 European wild boars from different Spanish regions (*n*=9) and from Iran (*n*=2) and Romania (*n*=2), Chinese Meishan (*n*=2) and Fengjing (*n*=1) domestic pigs, small Vietnamese pigs (*n*=6), Siberian wild boar (*n*=1) and pigs from a commercial cross (Youli) between Landrace and a Chinese-European composite line (*n*=2) were also included in the analyses.

### DNA extraction and sequencing

Genomic DNA from all animals was extracted from blood samples with a standard phenol: chloroform protocol (Sambrook et al. 1989), and used for sequencing and polymorphism identification. Four primer pairs were designed according to the reference sequence GenBank: AJ865080.1 of the porcine *LEP* gene to amplify 2520 bp of the intronic region between exons two and three (Table S1). The *LEPR* promoter region sequencing was defined in accordance with the pig *LEPR* gene structure described by Lee et al. (2008). According to the available *LEPR* gene sequence (GenBank:

FN677933.1), three primer pairs (Table S1) were designed to amplify 1266 bp, in three overlapped fragments.

PCRs were carried out in a 25 μl final volume containing 100 ng of DNA, 1 unit of polymerase (Biotools) or HotStart polymerase (Quiagen), specific buffer, 2 mM of dNTPs and 0.5 μM of each primer. The specific annealing temperature of each primer pair is shown in Table S1. The PCR reactions were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Warrington, UK). The PCR products were purified with the GFX™ PCR DNA purification kit (GE Healthcare, UK) according to the manufacturers' protocol. All products were sequenced using the 3100 BigDye® Terminator v3.1 Matrix Standard in a 3730 DNA Analyzer (Applied Biosystems Warrington, UK). The obtained sequences were edited and aligned using the EditSeq and MegAlign packages of the WinStar software for the identification of polymorphisms.

Mitochondrial *cytochrome B* (*Cyt B*) haplotypes (Fang and Andersson 2006) were determined by sequencing a fragment of 661 bp between sites 14,695 and 15,355 of the *Cyt B* gene as described in Alves et al. (2003) for characterizing the mitochondrial genetic origin of all the samples of the current study.

### Data analysis

Sequence overhangs were trimmed resulting in a total of 2480 bp aligned region for the *LEP* gene and 1200 bp for the *LEPR*. The different haplotypes of both genes were independently constructed with Phase v2.1.1 (Li and Stephens 2003) using default options.

Multiple alignments of all sequences per gene were performed with the Molecular Evolutionary Genetics Analysis version 5.05 (MEGA5) software (Edgar 2004; Tamura et al. 2011) and haplotypic dendrograms were inferred using the neighbour-joining model using the pair-wise distances (p-distance). To assess the robustness of the dendrogram topology, bootstrap resampling was carried out with 1000 replicates. The nucleotide variability of each one of the groups of pig populations was estimated calculating the number (*S*) and the proportion (*PS*) of segregating sites and the nucleotide diversity index ($\pi$). The haplotypes were reconstructed using Phase v2.1 (http://stephenslab.uchicago.edu/software.html). Haplotypic variation was measured by *i*) the number of observed haplotypes ($R_T$), *ii*) the haplotype richness corrected for sample size or expected number of different haplotypes ($R_R$) calculated using the rarefaction technique (El Mousadik and Petit 1996), and *iii*) the haplotype diversity metrics ($H_d$) as proposed by Nei and Tajima (1981) taking into account haplotype frequencies. The Hudson-Kreitman-Aguadé (HKA) neutrality test (Hudson et al. 1987) was applied to data from the sequenced regions of *LEPR* and *LEP* genes of Iberian pigs and European wild boars, assuming both proceed from a common ancestral population. The HKA test was conducted using the DnaSP v5software (Librado and Rozas 2009).

### *LEPR* gene expression conditional on promoter haplotype

The relative expression level of the *LEPR* gene (short and long isoforms, *LEPRa* and *LEPRb* respectively) used in the current study in 30 pigs from an F1 (Iberian x Landrace) x Landrace backcross corresponded to that determined in Pérez-Montarelo et al. (2012) in hypothalamus, liver, backfat and muscle tissues (*Longissimus dorsi* and diaphragm). The *LEPR* promoter region was also sequenced in these animals and their haplotypes were identified as mentioned before. The differential expression of this gene according to the haplotypes was investigated comparing the expression in those animals carrying haplotype *LEPR*H1 with the ones carrying the other haplotypes using the method proposed by Steibel et al. (2009).

## Results

### Nucleotidic variation

The analysis of mitochondrial *Cyt B* in the analysed samples showed the presence of the three described European haplotypes (E1, E2 and E3) and three (A1, A2 and A3) of the four Asian haplotypes (Fang and Andersson 2006), supporting the known introgression of east Asian genomes in several Western breeds (Duroc, Large White, Pietrain). No maternal Asian introgression was observed in Iberian pigs and European, Iranian and Romanian wild boars (Table 1).

The alignment of the sequences of the *LEP* gene intron 2–3 revealed a total of 81 polymorphisms in the 54 sequenced pigs. Four of these polymorphisms correspond to indels and the remaining 77 to SNPs. The number and the proportion of segregating sites and the nucleotide diversity for the different groups of pig breeds or populations are shown in Table 2. The highest nucleotidic variation for this gene was detected in the Asian breeds, with 65 segregating polymorphisms out of the 81 detected, followed by the Western domestic breeds (excluding the Iberian breed) that showed 58 segregating sites. Similarly, the highest nucleotide diversity index ($\pi$) was found in the Asian domestic breeds, with a value of 0.011, which is double those of Western pigs and European wild boars.

The alignment of the sequences of the promoter region of *LEPR* gene revealed 26 polymorphisms: two indels and 24 SNPs. The number and the proportion of segregating sites and the nucleotide diversity for the different groups of populations are shown in Table 2. The European domestic breeds (excluding the Iberian breed) showed the highest nucleotidic variation, with 21 segregating polymorphisms out of the 26 detected, followed by wild boars that showed 19 segregating

**Table 2** Nucleotide and haplotype variability of the analysed regions of *LEP* and *LEPR* genes in different Western and Asian porcine populations: number (*S*) and proportion (*PS*) of segregating sites, nucleotide diversity index ($\pi$), number of observed haplotypes ($R_T$), number of expected haplotypes estimated by rarefaction ($R_R$) and haplotype diversity ($H_d$)

| | Nucleotide variation | | | | Haplotype variation | | |
|---|---|---|---|---|---|---|---|
| | $2N$ | $S$ | $PS$ | $\pi$ (SE) | $R_T$ | $R_R$ | $H_d$ (SE) |
| *LEP* gene | | | | | | | |
| European wild boars* | 26 | 41 | 0.016 | 0.006 (0.0001) | 19 | 15.21 | 0.946 (0.040) |
| Iberian pigs | 24 | 38 | 0.015 | 0.005 (0.0001) | 19 | 16.00 | 0.948 (0.034) |
| Other Western domestic pigs | 38 | 58 | 0.023 | 0.005 (0.0001) | 29 | 16.96 | 0.980 (0.012) |
| Asian domestic and wild pigs | 20 | 65 | 0.026 | 0.011 (0.0001) | 13 | 13.00 | 0.905 (0.054) |
| Total | 108 | | | | 69 | | |
| *LEPR* gene | | | | | | | |
| European wild boars* | 26 | 19 | 0.016 | 0.004 (0.0001) | 11 | 9.55 | 0.883 (0.039) |
| Iberian pigs | 24 | 0 | 0 | 0 | 1 | 1.00 | 0 |
| Other Western domestic pigs | 38 | 21 | 0.017 | 0.006 (0.0001) | 11 | 8.04 | 0.716 (0.077) |
| Asian domestic and wild pigs | 20 | 18 | 0.015 | 0.004 (0.0001) | 7 | 7.00 | 0.811 (0.052) |
| Total | 108 | | | | 24 | | |

* wild boars from Spain, Romania and Iran

sites. Similar nucleotide diversity indexes, 0.004-0.006, were found in the different populations, except in the Iberian breed. It is remarkable that no nucleotidic variation was found in the promoter region of the *LEPR* gene in the analysed Iberian pigs.

## Haplotypic diversity

A total number of 69 segregating haplotypes were constructed for the *LEP* gene (Table S2). Most of them were found at low frequencies (ranging from 1/108 to 6/108). The observed $R_T$, $R_R$ and $H_d$ values of the different populations are shown in Table 2. The highest number of observed haplotypes was found in the group of Western domestic breeds, excluding the Iberian breed, and the lowest was detected in the Asian domestic breeds, although they showed the highest nucleotide diversity index. The haplotypic richness calculated, using the correction by sample size through rarefaction, and the estimated values of haplotypic diversity confirmed this result. Iberian pigs have similar haplotypic variation ($R_T$) and diversity ($H_d$) to the European wild boars, with higher values than the Asian group. In addition, the performed correction by sample size allowed us to determine that the haplotypic variation was higher in Iberian pigs than in wild boars. The results showed a high haplotypic variation in this gene, especially among European domestic pigs, but even within the Iberian breed that showed a total of 19 different haplotypes in the 12 individuals sequenced.

Based on the 69 haplotypes identified for *LEP* gene, a dendrogram was constructed (Fig. 1). Three major clades are distinguishable, named A to C. Clade A of the dendrogram comprises 53 haplotypes of European origin, including Iranian and Romanian wild boar haplotypes. Clades B and C contain the 16 haplotypes of likely Asian origin. Clade C is composed of the eight haplotypes detected only in the Vietnamese pig breed (*LEP*H63, H64, H65, H66, H67, H68 and H69), and clade B contains the four haplotypes detected in the Meishan animals (*LEP*H59, H60, H61 and H62), the unique haplotype identified in Feijing pig and shared with the Siberian wild boar and the Chinese-European cross Youli (*LEP*-H57), two haplotypes detected in Duroc pigs (*LEP*H55 and H56), and one haplotype identified in one European wild boar (H54). Although there is little consistency in the branches, two different subclusters could be considered in the European A clade: one containing a high representation of haplotypes from Iberian pigs and European wild boars, and another one mainly formed by other European domestic pig breeds excluding the Iberian breed, even though some Iberian and Spanish wild boars are also present in this subcluster. Moreover, some of the haplotypes are shared between Iberian, other Western domestic pigs and European wild boars (*LEP*H17 and *LEP*H51), between Iberian pigs and European wild boars (*LEP*H18), between Iberian pigs and other Western domestic breeds (*LEP*H19), and between European wild boars and other Western domestic breeds (*LEP*H39, *LEP*H40 and *LEP*H41).

A total of 24 segregating haplotypes were reconstructed for the *LEPR* gene (Table S3). Among the identified *LEPR* haplotypes, the *LEPR*H1 showed the highest frequency (equal to 51/108) while the others ranged from 1/108 to 8/108. The $R_T$, $R_R$ and $Hd$ for the different populations are shown in Table 2. Even though the observed number of haplotypes indicates that both, the Western breeds group and the European wild boars showed the highest haplotypic variation, the correction by rarefaction performed taking into account the different sample sizes of the populations ($R_R$) and the $H_d$ value, allowed to determine that the highest *LEPR* haplotypic variation is indeed found in wild boars.

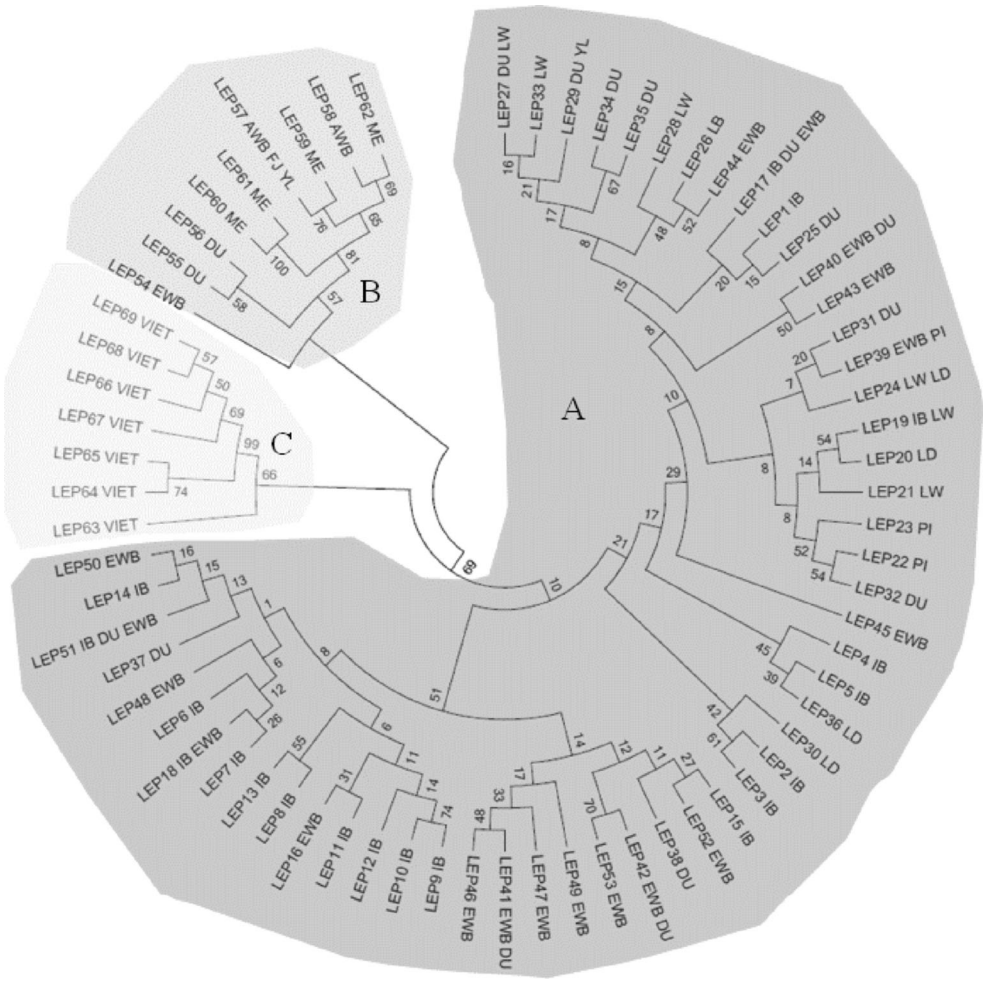


**Fig. 1** Neighbour-joining dendrogram of the 69 identified *LEP* haplotypes. Robustness of dendrogram tested by resampling of 1000 bootstrap replicates. Dark grey shadow covers European clade (**a**) and light grey shadows Asian clades (**b** and **c**)

The constructed dendrogram based on the 24 haplotypes identified for *LEPR* gene (Fig. 2) shows two main clades (A and B). The A clade of the dendrogram comprises mainly European pig breeds including Iberian and the European wild boars haplotypes (*LEPR*H1-11), except one haplotype present in the Fengjing sample (*LEPR*H20). The B clade contains Asian haplotypes distributed in two subclusters, one containing Chinese and Russian wild boar (*LEPR*H14, H16, H21 and H22) and a second subcluster containing the Vietnamese haplotypes *LEPR*H23 and H24. Within this B clade some European haplotypes are detected (*LEPR*H12, H13, H15, H17 and H18 detected in Large Black, Large White, Youli, Landrace and Duroc breeds). Finally, while most of the European wild boar haplotypes are in the European clade, there are two haplotypes (*LEPR*H12 and H13) that appeared within the Asian clade. It is noteworthy that 11 different haplotypes were identified in the 13 European wild boars, and seven different haplotypes appeared segregating in the nine Spanish wild boars included in this analysis. As shown in the dendogram, nine of these haplotypes were specifically identified in wild boars (*LEPR*H4, H5, H6, H7, H8, H9, H10, H12 and H13) and the remaining two are shared with European pig breeds (*LEPR*H1 and *LEPR*H2).

In agreement with the absence of nucleotidic variation found for this gene, all the Iberian samples analysed shared the same *LEPR*H1 haplotype. In order to check whether *LEPR* gene could undergo natural or artificial selection in this breed, a neutrality test (HKA, Hudson et al. 1987) was carried out. The HKA test was conducted comparing the sequences of both loci, *LEPR vs LEP*, and using European wild boars as a separate outgroup sharing a common ancestry (Table 3). The test result showed a significant departure from the expectations of neutrality ($\chi^2$ value=5.657; *P*-value<0.018) for *LEPR* gene in Iberian pigs.

The analysis of the promoter region sequence in the animals of the Iberian x Landrace backcross showed the presence of *LEPR*H1 haplotype in 42 out of the 60 sequences available from the 30 animals, and 18 carriers of four other haplotypes (*LEPRH25*, *LEPRH26; LEPRH27* and *LEPRH28*), which were not identified in the previous analysis (Table ESM3). The comparison of the *LEPR*a and *LEPR*b isoform expression levels of the *LEPRH1* carriers versus the remaining ones did not reveal significant results (Table 4).

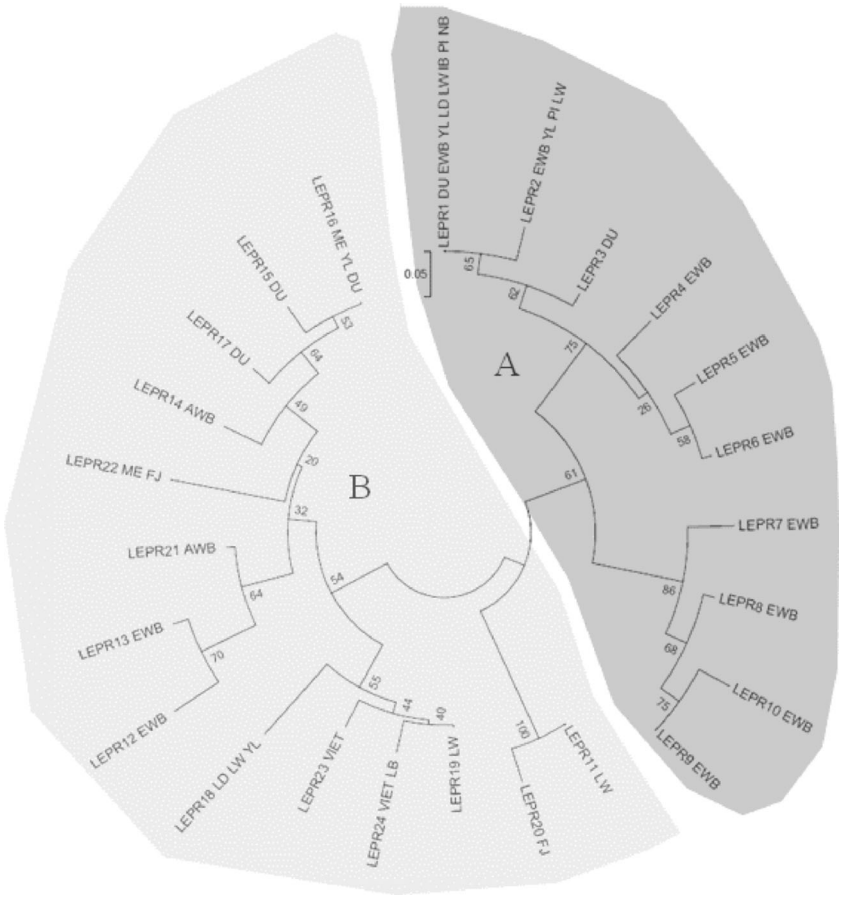


**Fig. 2** Neighbour-joining dendrogram of the 24 identified *LEPR* haplotypes. Robustness of dendrogram tested by resampling of 1000 bootstrap replicates. Dark grey shadow covers European clade (**a**) and light grey shadows Asian clade (**b**)

## Discussion

Previous studies indicated independent domestications of pigs in Asia and Europe (Larson et al. 2005, 2007), and dated the real shift in the pig populations to roughly 300 years ago, when Chinese pigs came to Europe combining two domestic pig ancestries isolated for more than 900 years. Millennia of diverging genetic, cultural and evolutionary pressure resulted in a remarkable genomic and phenotypic differentiation between pigs from both origins, although most of the genetic variation is now distributed within populations (Zhang and Plastow 2011). The introgression combined the larger body size of European pigs with the fatter body and faster early growth of the Asian ones (White 2011). Both well-known

**Table 3** Results of the Hudson-Kreitman-Aguadé neutrality test for *LEPR* vs *LEP* genes between European wild boars and Iberian pig breed

| | *LEPR* | *LEP* |
|---|---|---|
| Intraspecific polymorphism data (Iberian pig breed) | | |
| Segregating sites (obs): | 0 | 38 |
| Segregating sites (exp): | 2.25 | 35.75 |
| Total number of sites: | 1200 | 2480 |
| Sample size: | 24 | |
| Interspecific divergence (Iberian pigs *vs* European wild boars) | | |
| No. differences (obs): | 3.23 | 13.22 |
| No. differences (exp): | 0.98 | 15.47 |
| Total number of sites: | 1200 | 2480 |

Estimates of Theta (per nucleotide) in *LEPR*: 0.00050

Estimates of Theta (per nucleotide) in *LEP*: 0.00386

$\chi^2$ value=5.657 *P*-value=0.0174

**Table 4** Differential expression results of *LEPR* isoforms in hypothalamus, backfat, *Longissimus dorsi* and diaphragm muscles and liver: *LEPR*H1 minus the remaining *LEPR* haplotypes in a porcine Iberian x Landrace backcross

| Tissue | Isoform | Estimator | SE | *P*-value |
|---|---|---|---|---|
| Hypothalamus | *LEPRb* | 0.473 | 0.602 | 0.438 |
| Hypothalamus | *LEPRa* | 1.017 | 0.549 | 0.074 |
| Backfat | *LEPRa* | −0.856 | 0.604 | 0.164 |
| *Longissimus dorsi* | *LEPRa* | 0.379 | 0.369 | 0.310 |
| Diaphragm | *LEPRa* | 0.479 | 0.268 | 0.082 |
| Liver | *LEPRa* | 1.059 | 0.621 | 0.097 |

*LEPRa*: *LEPR* short isoform; *LEPRb*: *LEPR* long isoform, following Pérez-Montarelo et al. (2013) annotation

processes, domestication from different ancestors and introgression of Asian genomes in the European breeds during the XVIII and XIX centuries, are firstly illustrated in the present study by the distribution of *Cyt B* mtDNA haplotypes (Table 1). The different variants are unambiguous diagnostic markers of Asian and European mitochondrial lineages (Fang and Andersson 2006). It is noticeable that the analysed samples showed the presence of some rare *Cyt B* haplotypes: E3 in one of the Romanian wild boars, and A3, previously detected only in some East Asian wild boars and the Tamworth breed (Ramírez et al. 2009), is now identified in Vietnamese, Meishan, Duroc and Large White pigs.

The most polymorphic regions of the *LEP* and *LEPR* genes have been sequenced in a variety of pig breeds and wild boars, from Western and Asian origins, in order to analyse their nucleotidic and haplotypic diversity. These genes are involved in the regulation of appetite and energy balance and have a relevant impact on pig productive traits such as growth and fatness. The results obtained from the sequence analysis of the *LEP* intronic region showed three distinct clusters (Fig. 1), showing once more the genetic differences between East Asian and European haplotypes (Porter 1993; White 2011). Although European and Asian origins appeared as different clades in the dendrogram, some haplotypes (*LEP*54, H55 and H56) present in one European wild boar and two Duroc pigs are included in cluster B which groups Asian haplotypes, supporting evidence of the introgression of Asian genes into the genome of some European wild boars. Moreover, our *LEP* gene results support the existence of independent domestication events in two Asian regions according to the results based on complete mtDNA sequences reported by Wu et al. (2007), the small Vietnamese pigs grouped in cluster C could likely be derived from the Mekong region and the Asian (no Vietnamese) from the downstream region of the Yangtze River. In fact, the values of p-distances pointed out that the net distance (0.016±0.002) between B and C clusters grouping Asian haplotypes is greater than the net distances between these and the European A cluster: 0.009±0.001 and 0.009±0.001, respectively. The dendrogram also showed a typical clustering of the commercial lines around their respective breeds of reference (Ollivier 2009): the haplotypes of the Chinese-European composite line named Youli (*LEP*H29 and H57) clustered with European (*LEP*H29) and Asian (*LEP*H57) pig breeds.

A glance at Table 2 and Fig. 1 shows that 19 different *LEP* haplotypes were detected in the 13 EWB sequenced, 12 of them were specifically detected in this population, three are also present in Iberian pigs (*LEP*H17, H18 and H51) and the remaining four haplotypes are shared with other European pig breeds. However, these observations do not agree with previous studies that reported very low nucleotide diversity in European wild boars compared to domestic breeds such as described for the *FABP4* gene (Ojeda et al. 2006) and mtDNA (Larson et al. 2005; Fang and Andersson 2006). It is also worthy to mention the high haplotype diversity detected in the Iberian breed, in which a total of 19 haplotypes were identified in only 12 animals.

The dendrogram obtained for the haplotypes of *LEPR* promoter also support both the genetic differences between European and Asian breeds and the gene introgression of Asian into the European breeds. For instance, the fact that the Large Black sample shares haplotypes with Vietnamese pigs (*LEPR*-H24) is in agreement with the previously reported introgression of Asian origin into the Large Black population bases on *MC1R* alleles (Kijas et al. 1998). Again, the European wild boars revealed a high *LEPR* haplotypic diversity, the highest of the analysed population. A total of 11 *LEPR* different haplotypes were identified in the 13 analysed wild boars, nine of the 11 haplotypes were specifically detected in the wild boars, and only two were shared with European pig breeds (*LEPR*H1 and H2). Moreover, while most European wild boar haplotypes clustered with the European pig breed haplotypes, there are two haplotypes (*LEPR*H12 and H13) present in three heterozygous Spanish wild boars, separately clustered with the *LEPR*H21 haplotype, only detected in the Siberian wild boar. These three haplotypes form a dendrogram branch within the Asian B clade that could predate domestication. The identification of particular wild boar mtDNA haplotypes has been reported in a previous study (Giuffra et al. 2000), where wild boars from Italy clustered as a separate clade in the dendrogram.

The null *LEPR* variability observed in the Iberian breed is remarkable. This lack of haplotypic variability of the *LEPR* gene (1/24 haplotypes) contrasts with the results obtained for the *LEP* gene (17/69 haplotypes) and with previous studies that reported a high genetic variability of the Iberian breed in the *FABP4* (Ojeda et al. 2006) and *IGF2* genes (Ojeda et al. 2008) and using mtDNA (Alves et al. 2003) or microsatellites markers (Fabuel et al. 2004; Rodrigáñez et al. 2008; Gama et al. 2013). Iberian pigs represent an extremely fat phenotype, differing from leaner pig breeds by their high levels of voluntary feed intake, serum levels of circulating leptin and lipogenic potential. All these characteristics fit with a leptin resistance pattern in which leptin levels fail to reduce voluntary feed intake and obesity (Fernández-Figares et al. 2007; Ovilo et al. 2010). Some *LEPR* polymorphisms that reduce its gene expression and the LEPR ability to transmit leptin signal could be responsible for this phenotype, and the null *LEPR* genetic diversity in Iberian pigs could be explained by natural or artificial selection on this gene. To clarify this matter we performed an HKA neutrality test which is based on sequence data from *LEP* and *LEPR* polymorphisms in the Iberian breed and differences between pig groups for both genes. The results showed a significant departure from the expectations of neutrality for *LEPR* gene in Iberian pigs. Therefore, some mutations could have been favoured over others reducing the genetic variability around the selected target according to the

classical hitchhiking effect (Maynard Smith and Haigh 1974). The *LEPR*c.1987 T allele is fixed in the Iberian pig breed, and previous studies on the *LEPR*c.1987C>T SNP in experimental Iberian crosses support the association of this allele to a low *LEPR* mRNA expression, probably leading to a reduction in leptin signaling that is translated to greater growth and fatness (Ovilo et al. 2010).

In order to evaluate the potential effect of the *LEPR*H1 promoter haplotype, which is also fixed in Iberian pigs, on its gene expression, an additional analysis was conducted in an Iberian x Landrace experimental backcross that presents several *LEPR* haplotypes. The *LEPR*H1 carriers were tested against the remaining *LEPR* haplotype carriers in this material. The results did not reveal changes in gene expression conditional on promoter haplotypes, although a previous study in the same animal material revealed highly significant effects of several promoter polymorphisms, in particular for *LEPR*-g.35856G>A on the expression of different *LEPR* isoforms (Pérez-Montarelo et al. 2013). The examination of the alleles contained within the haplotypes in the 30 individuals showed that the allele reporting the highest expression difference in the previous study, *LEPR*g.35856G, last position in the haplotype, is now split in three different haplotypes (H1, H25 and H28), preventing the detection of the effect. These results suggest that the presence of *LEPR*H1 does not lead to gene expression changes in the quoted experimental cross, although the reduction in the genetic variability in Iberian pigs may indicate a selective sweep in the *LEPR* gene region in this breed.

The results of the present study show the high variability of the *LEP* gene in the different analysed populations and support once again the genetic divergence between Asian and Western pig breeds. A lower genetic variability of the *LEPR* gene promoter was observed within and between populations. Moreover, the Iberian pigs showed no variability for this *LEPR* region, contrasting with its high phenotypic, productive and genetic variation identified in previous studies (Alves et al. 2003; Fabuel et al. 2004; Ojeda et al. 2006; Ojeda et al. 2008; Rodrigáñez et al. 2008; Gama et al. 2013). Moreover, the results provided by the HKA test support a possible selection event of the *LEPR* gene in this breed potentially related with its fatness, high appetite and leptin levels, attributes that provide a good adaptation of Iberian pigs to the singular characteristics of their traditional extensive production system with a strong seasonal variation of the available feeding resources (López-Bote 1998).

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