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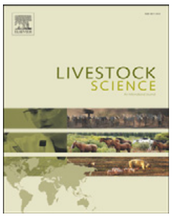

Morphological and genetic characterization of Spanish heavy horse breeds: Implications for their conservation

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ABSTRACT

Spanish heavy horses play a very important role in keeping up ecologically important rural areas. The interest in their conservation and characterization has increased over the last few years. The aim of this work is to contribute to the characterization of the endangered Spanish heavy horse populations in order to obtain useful information to implement conservation strategies for these genetic stocks. A total of 426 horses from six Spanish heavy horse populations corresponding to the four main heavy horse breeds (Hispano-Breton (HB) – with most animals located in three areas: Burgos (HBbu), León (HBle) and Palencia (HBpa) – Jaca Navarra (JN), Burguete (B) and the Cavall Pirinenc Català (CPC)) were analysed using 22 body measurements, 10 indices calculated from the body measurements and 16 microsatellite markers. From a morphological point of view, all the populations are clearly differentiated, except for the HBpa female population, which is located between the HBbu and JN populations. The HBle has greater body size than the other populations analysed, especially when compared to the JN. The genetic analysis provides us with information about the history of the analysed breeds. However, the most important point borne out by this analysis is the clear identification of different genetic backgrounds within the Spanish heavy horse populations. The overall information given in this work leads us to consider that the genetic scenario of the Spanish heavy horses is more likely to be due to 'ancestrally' different genetic backgrounds. Spanish heavy horse breeds resulted from the crosses between native mares and foreign stallions. The CPC population was the first in which this introgression occurred and the use of foreign stallions became more common. This work presents results based on neutral genetic variation, but also within- and between-population differences in morphological traits that have undergone artificial or natural (adaptive) selection. This information should be taken into account in future conservation strategies in order to contribute towards the efficiency of conservation measures. In this sense, the main problems are the small population size and genetic degeneration, with the consequent future loss of diversity. Therefore, the most immediate and effective conservation priorities would be (1) to avoid inbreeding within populations, (2) to increase the population size, and (3) to facilitate genetic exchange among the populations.

1. Introduction

Native Spanish horse breeds have traditionally been used for saddle and light draught because of their body shape. The interest in obtaining draught horses for agricultural and military purposes led to the introduction of heavy horses to increase body size. Among heavy breeds, Ardennes, Belgian Draught or Percheron stallions have been imported from the late 19th

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century onwards, to cross them with native Spanish mares (Aparicio, 1944; Pérez-Gutiérrez et al., 2008). Of the foreign horse breeds used, only Breton horses (mainly the Postier Breton variety) and their crosses adapted successfully to the Spanish environment and management conditions (Alonso, 1999; Pérez-Gutiérrez et al., 2008). Since the 1930s, the Spanish Military Cavalry services have encouraged the systematic crossing between Breton stallions and local mares of the Northern Spain mountain branches and the agricultural Levant to obtain heavy horses (Alonso, 1999). In the middle of the 20th century, the Spanish draught horse populations consisted of roughly 120,000 individuals (with well-defined populations in the Cerdaña area of Catalonia, Aragón, Navarra (Burguete), Northern Castilla-León, and also Valencia and Murcia in the Mediterranean area (Aparicio, 1944)). Since the 1960s, the Spanish Military Cavalry services have mainly used Breton stallions of the 'Spanish type', usually obtained from the Spanish draught horse populations, thus creating the Hispano-Breton (HB) breed (Alonso, 1999; Pérez-Gutiérrez et al., 2008).

During the latter part of the 20th century, the mechanisation of agriculture led to a marked decrease in the Spanish heavy horse population and some of them (such as those from Aragón and the Levant) disappeared, while those remaining were exploited on harsh mountain pastures to produce horse meat (Alonso, 1999).

At present, Spanish heavy horses play a very important role in keeping up the ecological importance of rural areas, and the interest in their conservation and characterisation has increased over the recent years. Three horse breeds, Jaca Navarra (JN), Burguete (B) and Hispano-Breton (HB), have recently established their studbooks, and are considered by the Government as endangered breeds. Moreover, a characterisation and conservation programme is being developed for the remaining Cavall Pirinenc Català (CPC) (Jordana et al., 2006). These populations make up about 90% of the total number of horses slaughtered in Spain nowadays.

The JN is a light draught breed located in the north of Navarra (South-Western Pyrenees). It is considered a remnant of the first crosses between the foreign heavy horses and the local mares, probably linked to the present Pottoka or Losino pony breeds (Solis et al., 2005). Despite its type differences, it has been included in genetic analyses as a Northern Celtic-Iberian native pony breed (Solis et al., 2005). Its studbook was established in 2001 and accounts for 595 registered animals.

The B is a medium-sized horse breed located in the north of Navarra. It is the result of the complete absorption of the local mares into the foreign heavy stallion stock. Its studbook was established in 1999 and has a total of 4007 registered animals.

The HB is mainly located in the north of Castilla-León, generally on the south side of the Cantabrian mountain range. Most animals are located in three different areas: Burgos (HBbu), León (HBle) and Palencia (HBpa). These populations have differences in their breeding programmes, and HBle is considered as the most representative of the breed (Alonso, 1999). This breed was officially recognised in 1960 and its studbook was established in 1998. It accounts for 6307 registered animals.

The CPC is what remains of the main Spanish heavy horse populations (the old Cerdaña horse). This population comprised over a half of the total heavy horses in Spain during the first half of the 20th century, and shows a greater influence of the foreign Breton stallions (Aparicio, 1944). At present, it is located mainly in the Central and Western Catalonian Pyrenees, with a total of 4513 animals.

The characterization of livestock breeds has traditionally been based on their type characteristics. However, their morphology is greatly affected by the selection processes, and this fact has led to the characterization of livestock populations via neutral markers (Druml et al., 2007; Zuccaro et al., 2010). Nevertheless, Ruane (1999) has pointed out that the relative value of genetic distance studies for breed characterization is limited, and other criteria, such as morphology, should be used. Besides the characterization of the different breeds, animal conformation allows us to define the productive use of each breed, and the analysis of its conformation traits is of great interest to meat producers (Alberti et al., 2008; Wolf and Jones, 2007).

The interest of breeders and researchers in Spain has mainly focused on the native Spanish saddle horses (Azor et al., 2007; Cervantes et al., 2008, 2009; Gómez et al., 2009a, 2009b; Valera et al., 2005). However, interest in the characterisation of draught horses in Europe, mainly for conservation purposes, has increased over the last few years (Druml et al., 2007; Pérez-Gutiérrez et al., 2008; Zuccaro et al., 2010).

Within the framework of breed conservation, genetic characterization is important in the conservation of breed integrity and is a prerequisite for managing genetic resources. The combination of genetic diversity and relationship information provides important baseline data for future breed conservation efforts, especially for critically endangered breeds.

The aim of this work is to contribute to the characterisation of the endangered Spanish heavy horse populations in order to obtain useful information for the implementation of conservation strategies for these genetic stocks.

2. Material and methods

2.1. Breeds and sampling

Six different populations of the main four Spanish heavy horse breeds (JN, B, HBbu, HBle, HBpa and CPC) were sampled. Their geographical location in Spain is described in Fig. 1. All of them are located in the north of Spain, with similar environmental conditions and composition of pasture land.

A total of 426 individuals (327 females and 99 males) belonging to these six populations were sampled for genetic and conformation analyses. The sampling of closely-related individuals (full and half sibs) was excluded, and only breeding stocks older than 4 years were included, because their bodies were fully developed (Druml et al., 2008; Molina et al., 1999). Foals and pregnant mares were also excluded. The composition of the sample is summarised in Table 1. This sampling proved very difficult due to the extensive production system of the heavy horse populations. The number of males sampled was lower than females due to the scarcity of males present in these endangered breeds, because the ratio of females to males is around 15:1.

All the animals came from different farms (at least 5 animals per farm) with similar management system (feeding,

Fig. 1. Geographical areas of location for the 6 Spanish heavy horse populations analysed.Where: CPC is Cavall Pirinenc Català, B is Burguete horse, HBbu is Hispano-Bretón from Burgos, HBle is Hispano-Bretón from León, HBpa is Hispano-Bretón from Palencia and JN is Jaca Navarra.

reproductive management, basic care, sanitary control, parasite control, etc.).

2.2. Body conformation analyses

The relationship between conformation and meat production has previously been shown (Alberti et al., 2008; Wolf and Jones, 2007). Therefore, a total of 22 body measurements (Fig. 2) was obtained using a standard measuring stick and a non-elastic measuring tape, directly from all the 426 individuals. They were taken by the same trained qualifier (across studs) from the left side, on a hard, level floor.

The measurements were: height at withers (HWi), height at point of hip (HHi), height at dock (HDo), height at point of buttock (HBu), height at hock (HHo), height of back (HBa), leg length (LL), depth of thorax (DTh), width of thorax (WTh), width of chest (WCh), external width of chest (eWCh, including the muscular development of this area), back width (BaW), loin width (LoW), hip width (HiW), rump width (RuW), bi-ischial width (BIW), body length (BLe), length of rump (LRu), thorax girth (TGi), cannon perimeter (CPe), back length (BaL) and loin length (LoL).

Using these body measurements, a total of 10 body indices were estimated to define the general conformation of these animals and the performance in different areas (meat production, riding, etc.) of these breeds:

- *Relative proportionality of thorax index* (RPTI): DTh/HWi. This shows the development of the thorax region related to the legs. The higher the value, the better the conformation for meat production.
- *Body index* (CI): Ble*100/TGi. This shows the conformation of the body (not including legs). Animals with shorter bodies are usually selected for meat production.
- *Proportionality index* (PVI): HWi/Ble. This shows the body proportionality, including legs.
- *Thoracic index* (TI): WTh*100/DTh. This shows the capacity of the thoracic cavity.
- *Dactyl-thoracic index* (DTI): CPe*100/TGi. This shows the animal's bone development, which is of great importance in the abattoir.
- *Dactyl-costal index* (DCI): CPe*100/WCh. This also shows the animal's bone development.
- *Relative thickness of the cane bone index* (RTCI): CPe*100/HWi. This shows the harmony between the body and bone development.
- *Pelvic index* (PI): HiW*100/LRu. This shows the shape of the croup, which is of great importance in animals for meat production.
- *Longitudinal pelvic index* (LPI): LRu*100/HWi. This shows the size of the croup. The higher the value, the better its conformation for meat production.
- *Transversal pelvic index* (TPI): HiW*100/HWi. This also shows the size of the croup relative to body height. Again, the higher the value, the better its conformation for meat production.

Table 1
Number of individuals sampled in each population of the Spanish heavy horses analysed.

Breed	Individuals sampled		
	Male	Female	Total
CPC	21	104	125
B	19	37	56
HBbu	14	57	71
HBle	14	33	47
HBpa	18	55	73
JN	13	41	54
Total	99	327	426

For explanation of legend: see Fig. 1.

All these statistical analyses for body measurements and indices were carried out using different procedures of the *Statistics for Windows v.6.0* package. The basic descriptive statistics and Duncan's multiple-range tests to difference groups were computed. Principal component analysis was carried out to determine the number of independent factors that account for most of the phenotypic variation in the body measurements. The body measurements were included as active traits, and the body indices as supplementary ones (represented but not included in the analysis). Males and females were analysed as separated groups. Only factors accounting for more variation than any individual type trait (eigenvalue ≥ 1) were retained. Additionally, the between-population squared Mahalanobis distance matrix (Mahalanobis, 1936) obtained from 22 body measurements, was computed as: $D^2_{ij} = (\ _i - \ _j)$ ‘$COV^{-1}(\ _i - \ _j)$; where D^2_{ij} is the distance between population i and j, COV^{-1} the inverse of the covariance matrix of measured variables x and $\ _i$ and $\ _j$ are the means of variable x in ith and jth populations, respectively, whose significance was verified by means of critical value $D\alpha$.

2.3. Genetic analyses

Due to the scarce genealogical information from the studbook of the breeds JN, B and HB, with only two known generations (the population of CPC does not yet have an officially recognised studbook), the population size (Ne) was estimated using the variance of the family size, following the equation proposed by Hill (1979), because in populations with little

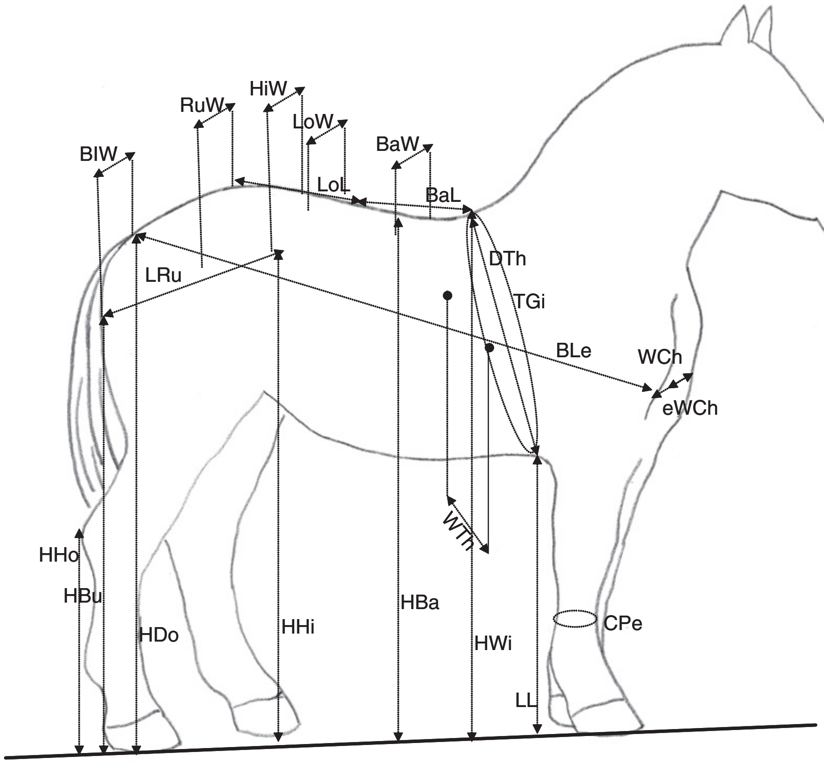

Fig. 2. Graphical representation of the twenty-two body measurements taken in the six Spanish heavy horse populations.Where: height at withers is HWi, height at point of hip is HHi, height at dock is HDo, height at point of buttock is HBu, height at hock is HHo, height of back is HBa, leg length is LL, depth of thorax is DTh, width of thorax is WTh, width of chest is WCh, external width of chest is eWCh, back width is BaW, loin width is LoW, hip width is HiW, rump width is RuW, bi-ischial width is BIW, body length is BLe, length of rump is LRu, thorax girth is TGi, cannon perimeter is CPe, back length is BaL and loin length is LoL.

pedigree information and without subdivision, this methodology seems to be the most suitable for estimating effective size:

$$\frac{1}{N_e}=\frac{1}{16ML}\left[2+\sigma^2_{mm}+2\left(\frac{M}{F}\right)\mathrm{cov}(mm,mf)+\left(\frac{M}{F}\right)^2\sigma^2_{mf}\right]$$
$$+\frac{1}{16FL}\left[2+\left(\frac{F}{M}\right)^2\sigma^2_{fm}+2\left(\frac{F}{M}\right)\mathrm{cov}(fm,ff)+\sigma^2_{ff}\right]$$

where: M and F are the number of males and females previously used as reproducers in a given period of time, L is the interval between generations, σ^2_{mm} and σ^2_{mf} are the variances of the number of the fathers' male and female descendants, σ^2_{fm} and σ^2_{ff} are the variances of the number of the mothers' male and female descendants, and $\mathrm{cov}(mm,mf)$ and $\mathrm{cov}(fm,ff)$ are the respective covariances.

As genealogical information was not available for these populations, molecular markers were analysed in order to perform the genetic characterization of the horse breeds. Blood samples were obtained in vacuum tubes containing EDTA. The total DNA was isolated from the whole blood sample using the salting-out procedure (Miller et al., 1988). Samples were genotyped for a set of 16 microsatellites recommended by the International Society for Animal Genetics (ISAG) Equine Genetics Standing Committee. The markers included in the analysis were those described by Azor et al. (2007). The microsatellites were amplified using fluorescent-labelled primers (StockMarks for horses, PE Applied Biosystems, Foster City, CA) following the PCR conditions given by Azor et al. (2007). The PCR products were frozen and stored until they were detected by capillary electrophoresis using an Applied Biosystems 3130 DNA sequencer. Allele sizes were determined after processing the raw data with the software packages GeneScan 3.7 and GeneMapper 3.7 using a LIZ 500 bp internal size standard (Applied Biosystems).

The above computations were performed using the programme MolKin 3.0 (Gutiérrez et al., 2005).

Among-population gene flow and genetic differentiation were assessed by computing the following between-population genetic parameters: molecular coancestry (f_{ij}), kinship distance (D_k) (Caballero and Toro, 2002), Standar Nei distance (Nei, 1972) and Reynolds distance (Reynolds et al., 1983). *F*-statistics described by Wright (1969), F_{IS}, F_{ST}, and F_{IT}, were also obtained.

The Genetix 4.2 programme (Belkhir et al., 2001) was used to compute the following parameters across loci and populations: allele frequencies, number of alleles per locus and observed heterozygosity (H_o). The genetic diversity of the analysed samples was assessed by computing the expected heterozygosity (H_e). The heterozygote deficiency within the population (F_{IS}) and the number of alleles per locus were corrected using Hurlbert's rarefaction method (Hurlbert, 1971).

Individual multilocus genotypes were also investigated by carrying out a canonical discriminant analysis, as implemented in the programme Genetix 4.2 (Belkhir et al., 2001) to obtain an unbiased test of population structure.

3. Results

3.1. Body conformation analyses

Mean values, standard deviations and the statistical significance of the differences at population level for the 22 body measurements are given in Table 2 (males) and Table 3 (females).

Table 2

Means for 22 body measurements (in metres) and 10 body indices, standard deviations and Duncan analysis for body measurements (in metres) obtained in males belonging to 6 Spanish heavy horse populations analysed.

Popul.	CPC		B		HBbu		HBle		HBpa		JN	
Var.	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
HWi	1.522^{bc}	0.051	1.480^{b}	0.050	1.523^{bc}	0.053	1.560^{c}	0.039	1.489^{b}	0.047	1.347^{a}	0.051
HBa	1.430^{b}	0.046	1.401^{b}	0.046	1.430^{b}	0.036	1.475^{c}	0.035	1.413^{b}	0.051	1.277^{a}	0.032
HHi	1.434^{de}	0.041	1.378^{bc}	0.062	1.417^{cd}	0.052	1.479^{e}	0.060	1.359^{b}	0.058	1.266^{a}	0.035
HBu	1.236^{bc}	0.037	1.266^{cd}	0.043	1.273^{cd}	0.051	1.306^{d}	0.042	1.211^{b}	0.056	1.120^{a}	0.047
HDo	1.381^{b}	0.026	1.387^{b}	0.048	1.409^{b}	0.040	1.456^{c}	0.041	1.369^{b}	0.053	1.255^{a}	0.044
LL	0.844^{c}	0.043	0.827^{bc}	0.051	0.788^{b}	0.043	0.850^{c}	0.056	0.739^{a}	0.066	0.784^{ab}	0.034
HHo	0.579^{b}	0.025	0.574^{b}	0.027	0.598^{bc}	0.035	0.608^{c}	0.029	0.590^{bc}	0.024	0.513^{a}	0.026
WCh	0.330^{b}	0.027	0.332^{b}	0.041	0.311^{b}	0.039	0.381^{c}	0.017	0.311^{b}	0.045	0.255^{a}	0.037
eWCh	0.531^{bc}	0.033	0.563^{c}	0.054	0.493^{b}	0.047	0.619^{d}	0.063	0.441^{a}	0.029	0.447^{a}	0.023
DTh	0.675^{b}	0.025	0.684^{b}	0.028	0.680^{b}	0.022	0.744^{c}	0.023	0.694^{b}	0.030	0.609^{a}	0.027
WTh	0.510^{b}	0.028	0.559^{c}	0.051	0.550^{c}	0.046	0.610^{d}	0.015	0.576^{cd}	0.032	0.448^{a}	0.025
BLe	1.635^{b}	0.046	1.599^{b}	0.068	1.619^{b}	0.049	1.710^{c}	0.031	1.587^{b}	0.057	1.424^{a}	0.066
BaL	0.345^{cd}	0.021	0.349^{d}	0.028	0.316^{bc}	0.028	0.365^{d}	0.019	0.309^{ab}	0.037	0.281^{a}	0.037
LoL	0.335^{ab}	0.025	0.322^{ab}	0.023	0.339^{b}	0.027	0.306^{a}	0.016	0.309^{a}	0.022	0.313^{a}	0.021
BaW	0.287^{b}	0.020	0.336^{cd}	0.057	0.306^{bcd}	0.037	0.341^{d}	0.022	0.301^{bc}	0.020	0.243^{a}	0.022
LoW	0.337^{b}	0.026	0.399^{c}	0.052	0.342^{b}	0.042	0.451^{d}	0.041	0.339^{b}	0.030	0.288^{a}	0.035
LRu	0.575^{c}	0.023	0.525^{b}	0.049	0.563^{bc}	0.016	0.594^{c}	0.019	0.530^{b}	0.019	0.455^{a}	0.061
HiW	0.512^{ab}	0.026	0.603^{c}	0.041	0.541^{b}	0.026	0.660^{d}	0.028	0.533^{b}	0.035	0.497^{a}	0.029
BIW	0.318^{b}	0.038	0.383^{c}	0.066	0.252^{a}	0.039	0.409^{c}	0.038	0.249^{a}	0.026	0.254^{a}	0.030
RuW	0.584^{bc}	0.023	0.613^{cd}	0.037	0.580^{bc}	0.030	0.639^{d}	0.076	0.549^{b}	0.018	0.504^{a}	0.026
TGi	1.952^{b}	0.061	2.015^{b}	0.121	1.970^{b}	0.069	2.215^{c}	0.114	1.936^{b}	0.083	1.723^{a}	0.067
CPe	0.251^{b}	0.015	0.274^{cd}	0.017	0.256^{bc}	0.019	0.288^{d}	0.019	0.277^{d}	0.029	0.217^{a}	0.016
RPTI	0.443^{a}	0.011	0.463^{bc}	0.017	0.447^{a}	0.019	0.477^{c}	0.024	0.466^{bc}	0.015	0.452^{ab}	0.010
CI	83.807^{c}	2.277	79.468^{abc}	2.963	82.224^{b}	2.280	77.385^{a}	4.983	82.023^{bc}	3.049	82.651^{c}	2.704
PI	0.931^{a}	0.031	0.927^{a}	0.041	0.941^{a}	0.029	0.912^{a}	0.026	0.939^{a}	0.034	0.947^{a}	0.029
TI	75.477^{a}	2.670	80.743^{b}	5.572	81.001^{b}	7.335	82.049^{b}	2.163	83.093^{b}	5.113	73.685^{a}	3.088
DTI	12.881^{ab}	0.625	13.590^{bc}	0.699	12.997^{ab}	0.826	12.977^{ab}	0.483	14.303^{c}	1.431	12.609^{a}	1.100
DCI	77.095^{a}	4.685	82.418^{ab}	8.834	83.945^{ab}	14.307	75.397^{a}	3.439	90.247^{b}	12.808	86.951^{ab}	14.435
RTCI	16.484^{a}	0.845	18.530^{b}	1.028	16.822^{a}	1.093	18.442^{b}	1.383	18.564^{b}	1.555	16.115^{a}	1.280
PVI	89.212^{a}	5.568	115.502^{c}	10.329	96.160^{ab}	6.163	111.189^{c}	4.378	100.746^{b}	8.378	110.930^{c}	15.070
LPI	37.795^{b}	1.605	35.523^{ab}	3.397	37.016^{b}	1.192	38.100^{b}	2.146	35.611^{ab}	1.556	33.786^{a}	4.775
TPI	33.663^{a}	1.557	40.758^{c}	2.323	35.579^{b}	2.290	42.333^{c}	2.241	35.789^{b}	1.961	36.849^{b}	1.514

[a,b,c,d,e] Different letters in the same row mean significant differences (Duncan test).

Where: Relative proportionality of thorax index is RPTI, corporal index is CI proportionality index is PI, thoracic index is TI, dactyl-thoracic index is DTI, dactyl-costal index is DCI, relative thickness of the cane bone index is RTCI, pelvic index is PVI, longitudinal pelvic index is LPI and transversal pelvic index is TPI. For explanation of legend, see Figs. 1 and 2.

Within heavy horses, the *HBle* population showed the highest average values, for males and females, especially for the traits related to body width: depth, width and perimeter of thorax, width of chest, body length and cannon perimeter. On the other hand, the *JN* had the lowest average values for all the body traits analysed, except for loin length. The *HBbu and HBpa* showed the lowest values for the body indices related to meat production, in males and females.

Females belonging to the CPC population were the second group by body size, followed by the B, HBbu and HBpa populations. For males, the order was B, CPC, HBbu and HBpa.

The length of the legs relative to the height at withers was evidenced by the relative proportionality of thorax index (the higher values are linked to shorter leg length and a better conformation for meat production). HBle is the population with the best values of this index in males and females, followed by B. In the same way, bone development is shown by the dactyl-thoracic, dactyl-costal and relative thickness of the cane bone indices. These show that HBpa is the population with the greatest bone development, whereas CPC and JN have the smallest.

The between-populations Mahalanobis distance matrix by their conformation traits is given in Table 4. The highest distances by their body measurements were found between the pair JN-HBle, for males (65.43) and females (67.48), whereas the least differentiated populations were HBbu and HBpa (17.58 and 7.52, for males and females respectively). The distances shown between the HBbu and the CPC and B populations were similar or lower than those computed with the other HB subpopulations. However, the HBle was similar to the B population, and significantly differentiated from the other heavy horses included in the other HB subpopulations.

The eigenvalues, i.e. the proportion of the total aggregate phenotypic variance of the analysed body traits, and the eigenvectors, i.e. the relative contribution of each individual trait to the identified factors, are given in Table 5. Only two components showed an eigenvalue equal to or higher than 1, accounting for 70.56% and 70.27% of the total variation of type traits for males and females, respectively. The first component accounted for 59.50% and 62.14% of the total variance for males and females, respectively.

The sign and magnitude of the eigenvectors allow us to interpret the factors. Most of the body measurements are related to Factor 1 (shape factor), although the bi-ischial width, related to facility to foaling, is related to Factor 2.

A two-dimensional projection of the variation patterns on the population level, identified with the principal component analysis, is shown in Fig. 3. Factor 1 (X-axis) differentiates the

Table 3
Means for 22 body measurements (in metres) and 10 body indices, standard deviations and Duncan analysis for body measurements (in metres) obtained in females belonging to 6 Spanish heavy horse populations analysed.

Popul.	CPC		B		HBbu		HBle		HBpa		JN	
Var.	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
HWi	1.499^{c}	0.053	1.436^{b}	0.043	1.484^{c}	0.041	1.553^{d}	0.040	1.448^{b}	0.051	1.332^{a}	0.040
HBa	1.417^{c}	0.048	1.367^{b}	0.049	1.408^{c}	0.045	1.466^{d}	0.046	1.368^{b}	0.051	1.258^{a}	0.031
HHi	1.414^{d}	0.051	1.349^{b}	0.056	1.383^{c}	0.039	1.444^{e}	0.037	1.346^{b}	0.054	1.238^{a}	0.039
HBu	1.217^{c}	0.053	1.229^{c}	0.057	1.225^{c}	0.042	1.277^{d}	0.038	1.188^{b}	0.044	1.094^{a}	0.054
HDo	1.380^{c}	0.054	1.357^{b}	0.048	1.377^{c}	0.040	1.437^{d}	0.039	1.363^{bc}	0.045	1.235^{a}	0.046
LL	0.826^{c}	0.041	0.814^{c}	0.059	0.811^{c}	0.048	0.868^{d}	0.044	0.734^{a}	0.035	0.757^{b}	0.045
HHo	0.564^{c}	0.032	0.551^{b}	0.028	0.562^{bc}	0.032	0.593^{d}	0.031	0.560^{bc}	0.023	0.497^{a}	0.025
WCh	0.302^{c}	0.027	0.310^{c}	0.031	0.281^{b}	0.027	0.353^{d}	0.024	0.271^{b}	0.032	0.240^{a}	0.031
eWCh	0.486^{d}	0.039	0.505^{e}	0.052	0.439^{c}	0.047	0.566^{f}	0.042	0.405^{b}	0.036	0.384^{a}	0.029
DTh	0.687^{b}	0.038	0.692^{b}	0.025	0.687^{b}	0.023	0.757^{c}	0.030	0.689^{b}	0.032	0.604^{a}	0.036
WTh	0.505^{b}	0.045	0.566^{d}	0.067	0.536^{c}	0.082	0.651^{e}	0.052	0.572^{d}	0.046	0.409^{a}	0.043
BLe	1.669^{c}	0.065	1.601^{b}	0.056	1.616^{b}	0.063	1.705^{d}	0.044	1.608^{b}	0.075	1.402^{a}	0.072
BaL	0.348^{d}	0.026	0.337^{c}	0.023	0.337^{c}	0.024	0.364^{e}	0.029	0.319^{b}	0.028	0.285^{a}	0.035
LoL	0.353^{c}	0.023	0.332^{ab}	0.027	0.337^{b}	0.027	0.321^{a}	0.028	0.324^{a}	0.028	0.327^{ab}	0.030
BaW	0.279^{b}	0.020	0.331^{d}	0.044	0.283^{bc}	0.029	0.318^{d}	0.031	0.293^{c}	0.026	0.221^{a}	0.025
LoW	0.338^{b}	0.025	0.383^{c}	0.042	0.332^{b}	0.023	0.416^{d}	0.030	0.327^{b}	0.032	0.279^{a}	0.031
LRu	0.580^{c}	0.032	0.539^{b}	0.026	0.545^{b}	0.031	0.582^{c}	0.030	0.540^{b}	0.032	0.441^{a}	0.044
HiW	0.565^{c}	0.028	0.599^{d}	0.049	0.561^{c}	0.030	0.668^{e}	0.028	0.539^{b}	0.033	0.494^{a}	0.024
BIW	0.311^{c}	0.032	0.358^{d}	0.069	0.256^{ab}	0.037	0.381^{e}	0.032	0.252^{a}	0.030	0.268^{b}	0.029
RuW	0.594^{d}	0.033	0.600^{d}	0.044	0.573^{c}	0.030	0.643^{e}	0.033	0.553^{b}	0.030	0.490^{a}	0.030
TGi	1.980^{c}	0.103	1.952^{c}	0.103	1.905^{b}	0.082	2.180^{e}	0.081	1.890^{b}	0.075	1.646^{a}	0.077
CPe	0.229^{b}	0.015	0.238^{c}	0.019	0.231^{b}	0.015	0.260^{d}	0.015	0.234^{bc}	0.013	0.195^{a}	0.011
RPTI	0.459^{ab}	0.022	0482^{cd}	0.015	0.463^{b}	0.014	0.487^{d}	0.020	0.476^{c}	0.017	0.454^{a}	0.028
CI	84.432^{c}	3.306	82.160^{b}	3.032	84.909^{c}	2.740	78.266^{a}	2.996	85.092^{c}	3.031	85.203^{c}	3.485
PI	0.898^{a}	0.028	0.898^{a}	0.031	0.919^{c}	0.030	0.911^{bc}	0.026	0.901^{ab}	0.031	0.195^{d}	0.044
TI	73.572^{b}	7.010	81.655^{d}	8.205	77.912^{c}	10.970	86.006^{e}	6.090	82.986^{d}	6.405	67.704^{a}	6.248
DTI	11.560^{a}	0.563	12.184^{cd}	0.913	12.107^{bcd}	0.531	11.927^{bc}	0.774	12.383^{d}	0.653	11.831^{b}	0.727
DCI	76.046^{a}	6.026	77.080^{a}	8.066	82.819^{b}	8.281	73.782^{a}	5.891	87.292^{c}	10.394	82.320^{b}	10.934
RTCI	15.263^{b}	0.813	16.536^{d}	1.200	15.539^{b}	0.815	16.726^{d}	0.846	16.165^{c}	0.906	14.609^{a}	0.852
PVI	97.564^{a}	5.502	111.455^{c}	9.755	103.071^{b}	7.524	114.930^{d}	4.740	99.974^{a}	7.437	112.913^{cd}	11.663
LPI	38.702^{c}	1.616	37.528^{b}	1.741	36.758^{b}	1.854	37.496^{b}	1.991	37.310^{b}	1.777	33.148^{a}	3.239
TPI	37.701^{a}	1.682	41.723^{b}	3.004	37.797^{a}	2.043	43.028^{c}	1.607	37.218^{a}	2.069	37.091^{a}	1.598

[a,b,c,d,e] Different letters in the same row mean significant differences (Duncan test).
Where: Relative proportionality of thorax index is RPTI, corporal index is CI proportionality index is PI, thoracic index is TI, dactyl-thoracic index is DTI, dactyl-costal index is DCI, relative thickness of the cane bone index is RTCI, pelvic index is PVI, longitudinal pelvic index is LPI and transversal pelvic index is TPI. For explanation of legend, see Figs. 1 and 2.

body size, with the highest separation between HBle and JN populations, in both males and females. Individuals belonging to the CPC, B, HBbu and HBpa populations did not show clear patterns of differentiation regarding body size. On the contrary, Factor 2 differentiates the CPC population from the rest.

3.2. Genetic characterization

The effective sizes obtained by family variance (Ne, without the inclusion of a possible subdivision of the population and usually overestimated) were 36.9 for JN, 226.1 for B, 130.1 for HBle, 65.4 for HBbu, 15.5 for HBpa and 273.6 for CPC (Table 6).

Table 4
Between-population Mahalanobis distance matrix obtained from 22 body measurements for the males (above diagonal) and females (below diagonal), of the 6 Spanish heavy horse populations analysed.

Populations	CPC	B	HBbu	HBle	HBpa	JN
CPC	–	49.79	28.69	51.22	62.65	50.23
B	20.64	–	25.63	25.98	31.37	34.49
HBbu	10.58	15.18	–	39.64	17.58	31.35
HBle	32.64	19.62	30.68	–	45.27	65.43
HBpa	22.13	22.25	7.52	43.08	–	45.31
JN	32.27	30.27	22.69	67.48	27.62	–

All distance pairs were statistically significant for $p<0.0001$.
For explanation of legend: see Fig. 1.

The parameters characterising within-population genetic variability are shown in Table 7. The expected heterozygosity (He) varied from 0.700 in HBpa to 0.789 in JN. The parameter F_{IS} was positive and high for all the heavy horse populations, showing a clear heterozygote deficiency within the populations, varying from 7.5 % (HBpa) to 20.7% (HBbu).

The values computed for F_{ST}, F_{IS}, F_{IT}, mean kinship distance (D_k), Nei's standard distance (D_N) and Reynold's distance (D_R) for the whole population using microsatellite markers were 0.026, 0.160, 0.182, 0.444, 0.086 and 0.015, respectively.

The between-population molecular coancestry (f_{ij}) and kinship distance (D_k) matrices are given in Table 8. The B, HBbu, HBle and HBpa populations had the highest f_{ij} values above 0.21, varying from 0.210 for the pair HBbu–HBle to 0.243 for the pair B-HBpa. Within the heavy horse populations, the lowest f_{ij} values were found for the JN breed and, especially, for the CPC population (0.192). In line with these findings, the lowest levels of D_k were also found between the B, HBbu, HBle and HBpa populations (below 0.48). These four populations have slightly higher values of Dk than the JN population, especially

Table 5
Eigenvalues proportional to the total phenotypic variance explained and eigenvectors of the first two main factors identified with the principal components analysis for the 22 body measurements analysed in 6 different populations of Spanish heavy horses.

	Males		Females	
	Factor1	Factor2	Factor1	Factor2
Eigenvalue	13.090	2.432	13.670	1.789
% variance	59.502	11.057	62.135	8.130
Cumulative variance	59.502	70.558	62.135	70.265
HWi	**−0.803**	0.480	**−0.896**	0.306
HBa	**−0.836**	0.439	**−0.875**	0.302
HHi	**−0.811**	0.356	**−0.840**	0.369
HBu	**−0.877**	0.099	**−0.827**	0.213
HDo	**−0.889**	0.296	**−0.876**	0.301
LL	−0.385	−0.273	**−0.617**	−0.019
HHo	**−0.708**	0.420	**−0.772**	0.239
WCh	**−0.773**	−0.284	**−0.808**	−0.268
eWCh	**−0.760**	−0.458	**−0.805**	−0.331
DTh	**−0.852**	0.176	**−0.827**	−0.016
WTh	**−0.836**	0.078	**−0.721**	−0.213
BLe	**−0.892**	0.210	**−0.872**	0.255
BaL	**−0.707**	−0.262	**−0.708**	0.046
LoL	−0.183	0.274	−0.235	0.479
BaW	**−0.772**	−0.333	**−0.718**	−0.301
LoW	**−0.770**	−0.509	**−0.809**	−0.409
LRu	**−0.790**	0.174	**−0.825**	0.251
HiW	**−0.760**	−0.347	**−0.832**	−0.323
BlW	**−0.600**	**−0.672**	−0.576	**−0.545**
RuW	**−0.847**	−0.193	**−0.876**	−0.100
TGi	**−0.947**	−0.014	**−0.933**	−0.108
CPe	**−0.768**	0.093	**−0.798**	−0.086

Eigenvectors greater than 0.600 are in bold.
For explanation of legend: see Table 2.

for the pair JN-HBpa (0.457). The differentiation between the CPC population and the other heavy horse populations was very marked.

Fig. 4 shows a two-dimensional plot summarising the dispersion of the genotyped individuals, after performing a correspondence analysis, and their multi-locus genotypes. The X-axis allows us to differentiate the JN from the other heavy horses while the Y-axis allows us to ascertain genetic differences within HB horses, especially HBpa from HBbu and HBle, and CPC horses. The most closed populations are HBbu and B.

4. Discussion

This work includes a comprehensive morphological and genetic characterization of a sample of Spanish heavy horse breeds. The morphological and genetic parameters have produced a wealth of varied information that should be carefully interpreted within the conservation programme of these breeds.

From a morphological point of view, based on this data set, the JN is clearly differentiated from the other Spanish heavy horses. Body measurements reflect the influence of both the genetic background and artificial selection. In this case, the JN is expected to be a direct result of the initial crosses between Celtic-Iberian mares and foreign heavy stallions (Aparicio, 1944). This is the reason why previous studies have included this breed as a member of the Northern Iberian ponies (Solis et al., 2005). However, the JN individuals form a light draught breed easily distinguishable from the other heavy horses analysed (Fig. 3), because their mean body measurements exceed those usually accepted for Celtic-Iberian ponies. Note, for example, that the bay-Asturcón, a semi-feral Celtic-Iberian pony population which was recently included in the Asturcón breeding programme (Royo et al., 2007), had an average HWi, DTh and Ble of 1.23 m, 0.57 m, and 1.26 m, respectively.

The HBle has also shown a clear differentiation in body measurements, with a greater body size than the other heavy horse populations analysed. This population also showed more adequate body indices related to meat production (body index, pelvic index, longitudinal pelvic index and transversal pelvic index), which could be caused by the continuous selection for meat quality. It has also shown a clear differentiation with the JN breed, with a Mahalanobis distance three-fold higher to that of B for males and females (65.43 and 67.48, respectively), (Table 4). Also, the Mahalanobis distance between the HBle and the other HB populations is statistically significant.

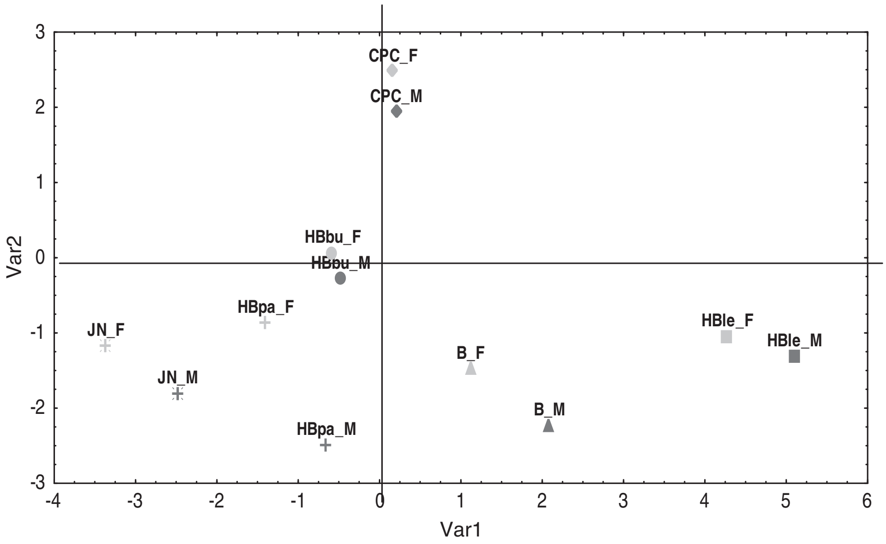

Fig. 3. Two-dimensional plot summarising the patterns of variation of the populations measured for twenty-two body traits using the two main factors identified with the principal component analysis.Where: CPC is Cavall Pirinenc Català, B is Burguete horse, HBbu is Hispano-Bretón from Burgos, HBle is Hispano-Bretón from León, HBpa is Hispano-Bretón from Palencia, JN is Jaca Navarra, F is female and M is male.

Table 6
Effective sizes using family size variance (Ne) in the 6 Spanish heavy horse populations analysed.

	CPC	B	HBbu	HBle	HBpa	HBwhole	JN
Number of males	537	439	121	240	28	389	60
Number of females	3976	3568	1726	3587	605	5918	535
Total population	4513	4007	1847	3827	633	6307	595
N_e	273.6	226.1	65.4	130.1	15.5	97.8	36.9

For explanation of legend: see Fig. 1.

The HBle has been long considered the most representative subpopulation of the HB breed and its maintenance and improvement has received economic funding and technical support. Traditionally, the most outstanding HB stallions have been located in this area, and Alonso (1999) has also argued that the HBle individuals are bred in areas with more favourable management, environmental and pasture conditions, thus allowing for the full expression of their genetic background.

The least differentiated populations were HBbu and HBpa, for males and females (17.58 and 7.52, respectively). The CPC and the B populations were in intermediate positions with the HB subpopulations.

Documentation of existing genetic resources, including the description of the population phenotypic characteristics, performance, cultural importance and genetic uniqueness is one of the main areas of livestock conservation activities (Duchev and Groeneveld, 2006; Ruane, 1999). Description of genetic diversity can also inform us about further sustainable intensification of animal production. These include incorporating this information into managing phenotypic characteristics, performance, quantitative trait loci development, cultural importance and preserving the genetic uniqueness of the remaining populations. The effective population size has a direct relationship with the rate of inbreeding, fitness and the amount of genetic variation lost due to random genetic drift (Cervantes et al., 2011), and thus, is also used as a criterion for characterising the risk status of livestock breeds (Duchev et al., 2006). This risk status can warn us about the need for action and how urgent this action should be. The risk level quantifies the probability of population extinction within the present circumstances and expectations (Gandini et al., 2004). Table 6 shows that the populations of JN, Hbbu and Hbpa are in risk of extinction, unless a suitable policy for the genetic conservation of the populations is not implemented.

The genetic analyses provide us with information about the history of the analysed breeds. The populations analysed showed high levels of Nei (1987) gene diversity. In addition, there were positive, high F_{IS} values, which reveal significant heterozygote deficiency (Table 7). This situation has been reported to be typical of base populations which undergo a recovery programme. As noted in the Asturcón pony (Royo et al., 2007) and in other species (Álvarez et al., 2008; Jordana et al., 2010), the individuals chosen to be founders of a breed are usually obtained at different genetically-isolated locations, probably leading to the fixation of different alleles (positive F_{IS} values). However, on the whole, these individuals capture the genetic remnant variability in the population before the bottleneck, which is mainly shown by the high number of heterozygosities expected and the average number of alleles per locus found (it should be noted that the conservation programmes of the analysed populations started very recently).

However, one major feature revealed by the genetic analyses is the clear identification of four different genetic backgrounds within Spanish heavy horses. The correspondence analysis (Fig. 4) allows us to separate the CPC, JN and HBpa populations from the other populations analysed, which tended to cluster in an intermediate position with respect to HBbu, HBle and B. Two alternative hypotheses can explain this situation: a) the isolated populations have recently diverged from the rest of Spanish heavy horses due to an intense population bottleneck and reproductive isolation; and b) the genetic differences found correspond to 'ancestrally' different genetic backgrounds.

The overall information given here leads us to consider that the genetic scenario of Spanish heavy horses is more likely to be due to the second hypothesis. This statement can be supported by the information provided by the parameters based on molecular coancestry. First, it should be noted that the within-population molecular coancestry values computed for the CPC and JN populations are the lowest, thus highlighting that individuals forming these breeds have the lowest levels of genetic identity among the populations studied (Table 7). Second, a more in-depth study of the D_k and f_{ij} matrices (Table 8) may

Table 7
Within-population and expected heterozygosity (H_e), heterozygote deficiency within population (F_{IS}), number of alleles per locus (k), and molecular coancestry (f_{ii}), computed using a set of 16 microsatellite markers belonging to the 6 Spanish heavy horse populations analysed.

Population	H_o	H_e	F_{IS}	k	f_{ii}
CPC	0645	0.784	0.176	9.3	0.217
B	0,634	0.758	0.163	8.5	0.242
HBbu	0,612	0.771	0.207	8.3	0.227
HBle	0,647	0.753	0.144	7.8	0.244
HBpa	0,646	0.700	0.075	6.9	0.300
JN	0,653	0.789	0.175	8.9	0.208
Whole population	0,640	0.782	0.156	8.3	0.240

For explanation of legend: see Fig. 1.

Table 8
Between-population paired molecular coancestry (fij: above diagonal) and pairwise kinship distance (Dk: below the diagonal) for the 6 Spanish heavy horse populations analysed.

Population	CPC	B	HBbu	HBle	HBpa	JN
CPC		0.211	0.206	0.207	0.223	0.192
B	0.469		0.220	0.223	0.243	0.201
HBbu	0.479	0.468		0.210	0.236	0.196
HBle	0.470	0.457	0.475		0.230	0.194
HBpa	0.454	0.437	0.449	0.447		0.218
JN	0.483	0.477	0.488	0.481	0.457	

For explanation of legend: see Fig. 1.

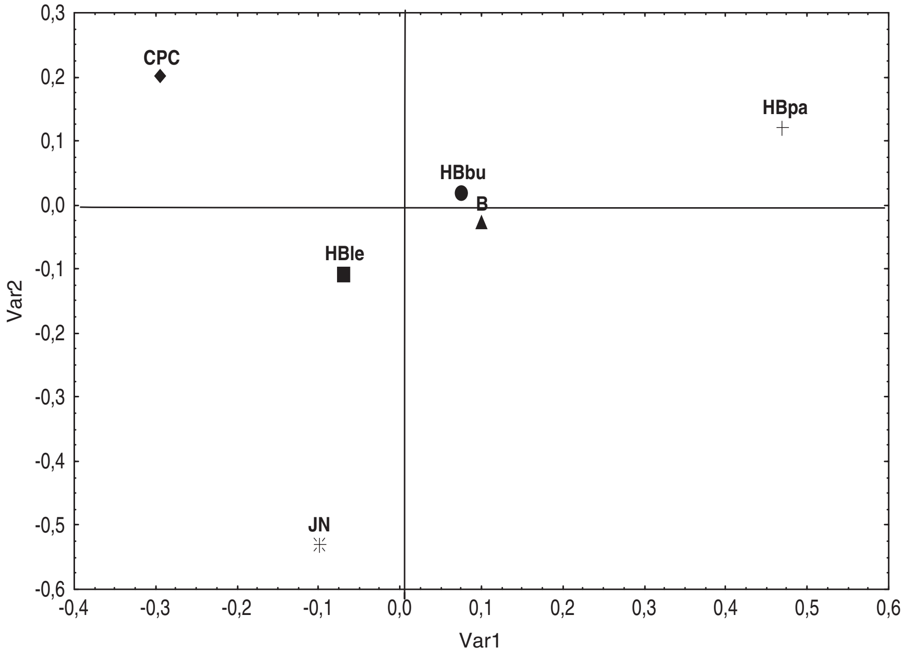

Fig. 4. Correspondence analysis using multi-locus genotypes in the Spanish heavy horse breeds analysed.

help to assess whether differentiation among breeds is recent or remote in origin. The two terms of the formula used to compute the kinship distance, $D_k = ([s_i + s_j]/2) - f_{ij}$ (Caballero and Toro, 2002) summarise the between-population differentiation after separation (in the term $(s_i + sj)/2$), corrected by the genetic identity of the populations at the moment of the original (ancestral) metapopulation fission (f_{ij}; Eding and Meuwissen, 2001). In the particular case of absence of differentiation after the separation of populations, the kinship distance would be $D_k = 0.5 - f_{ij}$. A simple comparison of two-dimensional scaling plots, constructed using D_k and $0.5 - f_{ij}$, illustrates whether the present differentiation of the populations is exclusively dependent on the differentiation at the time of metapopulation fission or not (Eding et al., 2002). The between-population molecular coancestry matrix (f_{ij}), shown in Table 8, shows that the HBpa population has the highest value compared with the rest of the population analysed.

Identifying the origin of these genetic backgrounds is not an easy task. Spanish heavy horse breeds resulted from the crosses between native mares and foreign stallions. The Catalonian heavy horse population was the first in which this introgression occurred and, mainly for geographical reasons, the one which involved a more intense use of foreign stallions (Aparicio, 1944). To test if the CPC background corresponds to that of the foreign individuals, we used genotypes of French stallions (Breton and Comtois breeds) used as reproducers in the CPC population location area. The re-run correspondence analysis showed that the foreign stallions clustered within the HB cluster, thus leading us to reject this hypothesis.

4.1. Implications for conservation

The molecular variability and the genetic structure of populations have crucial implications for management and conservation decisions. This information should therefore be taken into account when taking management decisions for the conservation of Spanish heavy horse breeds.

From a general point of view, it seems that the genetic distances between populations cannot contribute to a conservation programme by themselves. Nevertheless, because of the reduced size of the populations, the relationships between them could be of great use, in extreme situations, for increasing the Ne of close populations and thus avoiding extinction. The accumulated distances show the possibility of a different composition of the initial genetic backgrounds or the selection of a different type of animal. In this sense, the JN and CPC, which have very different conformations, are also very different from a genetic point of view. Thus, we can conclude that they have different origins. From a conservationist point of view, it is essential to avoid separations and to favour the tendency to maximize Ne.

Accurate estimates of Ne are central to developing suitable conservation strategies, because the Ne of a population is often many times smaller than its census size (N), with the Ne/N ratio averaging from just 0.02 (HBpa) to 0.06 (JN and CPC). Therefore, considering the population's observed genetic structures, the main problems are small population size and genetic degeneration, with the consequent future loss of diversity. The most immediate and effective conservation priorities for maintaining genetic variation would be (1) to avoid inbreeding within populations with a suitable mating plan, (2) to increase the population size, especially in the JN population, and (3) to facilitate genetic exchange among the populations, especially in the HB and CPC populations.

It would be also of great interest to implement conservation and selection criteria (by conformation traits, because of their relation with meat production) in order to maintain genetic variability and to improve the characteristics of the animals with a sustainable model. The main objective of the breeding programmes is the selection of the future reproducers for their conformation characteristics, while limiting the increase of kinship in the population using a dynamic methodology known as “optimal contribution methodology” (Caballero et al., 1996). This methodology also allows us to lower the morphological variability for the important characteristics (uniform commercial product), reducing it to a minimum genetic variability.

Thus, it certainly would be of interest to collect information related to pedigree and performance tests for meat production, in order to ensure suitable management of the conservation and selection programmes and to avoid high economic costs in the future.

Conflict of interest statement

None.

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