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Short communication. Mitochondrial DNA diversity of the founder populations of the Asturcón pony

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Abstract

A 380 bp fragment of the horse D-loop region was analysed in 42 founder mare samples of the bay-coated Asturcón pony obtained in three different and reproductively isolated mountainous areas of Western Asturias: range of "La Bobia" (20), range of "Carondio" (13) and range of "El Aguión" (9). These sequences were compared with the information provided by 37 founder matrilines of black-coated Asturcón assigned to the range of "Sueve" (26) and the "out-of-Sueve" (11) founder populations. The aim of this research was to ascertain the differences in founder mtDNA diversity between the two strains of the Asturcón pony and if such differences have geographical consistency. The 79 sequences analysed gave 16 different haplotypes defined by 33 polymorphic sites. The two Asturcón strains shared eight haplotypes that gathered 76% and 81% of the samples available in bay-coated and black-coated Asturcón, respectively. Both haplotypic (0.027 ± 0.006) and nucleotide (0.021 ± 0.011) diversity were higher in the bay-coated than in the black-coated Asturcón (0.024 ± 0.005 and 0.016 ± 0.009, respectively). AMOVA analyses failed in assessing any statistically differentiation among Asturcón geographical populations or strains. Most genetic variability is due to the individuals (estimates varying from 96.34% to 99.81%). Differentiation among strains or population took low and non-significant values. Differentiation between Asturcón pony strains using mtDNA marker would not have clear support. The two strains of the Asturcón pony breed likely derive from the same ancestral mare population.

Additional key words: D-loop; genetic variability; population structure; horse.

The Asturcón pony has an iconic status in Asturias and it is considered as one of the most ancient representatives of the Iberian horses (García-Dory, 1980). Representative individuals of this breed are shown in the Suppl. Fig. 1 [pdf online]. After a dramatic population bottleneck occurred in the mid-20th century, the recovery of the Asturcón pony breed started during the 1970s. The implementation of the conservation program caused controversy: the animals used as founders of the recovery programme were obtained from the black-coated population managed in semi-feral conditions in the range of Sueve (eastern Asturias; García-Dory, 1980; Royo *et al*., 2007; Álvarez *et al*., 2011). The initial process of recovery of the breed is well documented (García-Dory, 1980; Álvarez-Llana, 1995; Álvarez-Sevilla, 1995). During the early 1990s, a few additional black-coated mares from the surroundings of the Sueve's area and the Picos de Europa area, bordering Cantabria and León, were included in the Asturcón studbook as founders (Royo *et al*., 2007; Goyache *et al*., 2011). Finally, the total number of black-coated founder mares giving offspring included in the recovery program of the Asturcón pony was 50 (Royo *et al*., 2007; Goyache *et al*., 2011).

Recently, the breeders association (ACPRA) included in its breeding programme bay-coated Asturcón individuals recovered in Western Asturias (Royo *et al*., 2007; Álvarez *et al*., 2011). The recovery strategy applied was different from that carried out for the black-coated Asturcón: ACPRA decided to enlarge as much as possible the founder population size to allow further selection for type quality.

Yet, the controversy on the recovery programme remains. Mitochondrial (mt) DNA analyses reported

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that the main matrilineal composition of the Asturcón pony belongs to the Jansen *et al*.'s (2002) haplotypic family D (Royo *et al*., 2005a). However, the mtDNA haplotypic variability in the black-coated Asturcón is much larger than that assessed in the bay-coated Asturcón (Royo *et al*., 2007; Álvarez *et al*., 2012) suggesting that the founder population of the black-coated Asturcón has a larger influence of foreign horse breeds.

Here the mtDNA haplotypes assigned to the founder matrilines of black-coated Asturcón analysed in Álvarez *et al*. (2012) are compared with new sequences obtained from founder bay-coated Asturcón individuals sampled in three different and reproductively isolated areas of Western Asturias. The black-coated Asturcón pony matrilines were assigned to either a "Sueve" or to an "out-of-Sueve" area in accordance with Royo *et al*. (2005b). The aim of this research was to ascertain the differences in founder mtDNA diversity between the two strains of Asturcón pony and if such differences have geographical consistency. Inferences on the ancestral mtDNA composition of the Asturcón pony will also be made.

Sampling locations are illustrated in Fig. 1. Blood samples were obtained from a total of 42 bay-coated Asturcón founder matrilines sampled in three different, geographically isolated, areas. Twenty matrilines were sampled in the range of "La Bobia"; thirteen matrilines were sampled in the range of "Carondio"; and, finally, nine matrilines were sampled in the range of "El Aguión". Within Western Asturias, the "La Bobia" and the "El Aguión" areas have the most westerly and the most easterly locations, respectively, while the "Carondio" area has a central location. The 37 founder matrilines of black-coated Asturcón analysed in Álvarez *et al*. (2012) were assigned to a "Sueve" founder population (26) and to an "out-of-Sueve" founder population (11) according to Royo *et al*. (2005b). The "Sueve" population gathered those founder mares recovered in different management units within the range of Sueve. The out-of-Sueve population included those mares recovered during the early 1990s out in the lowlands of Eastern Asturias and the area of Picos de Europa.

Total DNA was isolated from blood samples following standard procedures (Sambrook *et al*., 1989). The laboratory methods used to amplify a 380 bp region of the horse mtDNA are described in full in Álvarez *et al*. (2012). Mitochondrial DNA sequences were aligned using the program CLUSTAL implemented in the

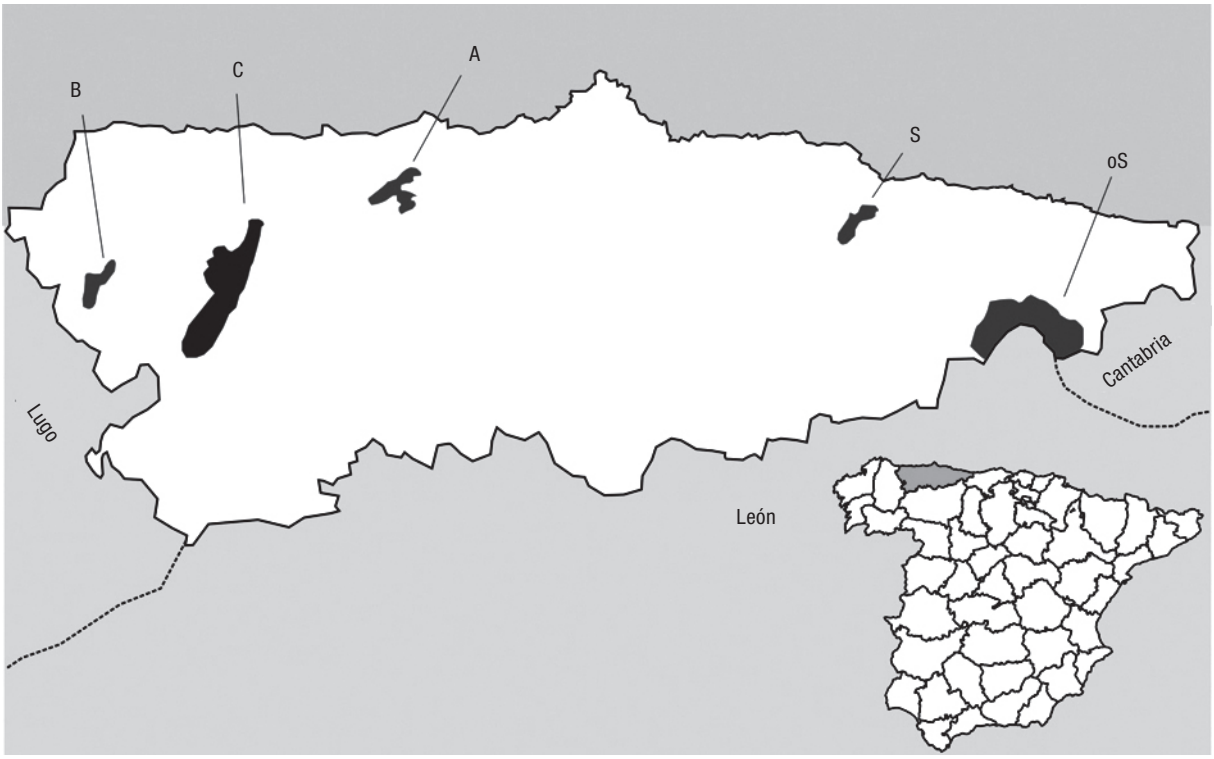

Figure 1. Geographical areas in which the Asturcón pony populations analysed were sampled. Abbreviations mean the following: "La Bobia", B; "Carondio", C; "El Aguión", A; "Sueve", S; and "out-of-Sueve", oS.

software MEGA 5.03 (Tamura *et al*., 2007; available at http://www.megasoftware.net/). Analyses were restricted to 380 bp. The nucleotide positions were numbered following Acc. No. X79547 (Xu & Arnason, 1994). According to the polymorphic sites, the identified haplotypes were assigned to the haplogroups defined by Jansen *et al*. (2002; see Table 2 of that paper). Definition of haplotypes has been done following Álvarez *et al*. (2012). The haplotypes that were not identified in such manuscript are here numbered starting from haplotype (Hap) 16. To derive the between-haplotypes phylogenetic relationships we constructed a median-joining network (MJ network) using the Network 4.5.1.0 program (Bandelt *et al*., 1999), available at http://www.fluxus-engineering.com/. The software Arlequin 3.5 (Excoffier & Lischer, 2010) was used to assess: a) haplotype and nucleotide diversity at the population level (Table 1); and b) population structure via AMOVA analyses. Statistical confidence for computed values was estimated by permutation analysis using 1000 replicates.

The 79 sequences analysed gave 16 different haplotypes including those 11 previously identified in black-coated Asturcón by Álvarez *et al*. (2012) (Table 1). The bay-coated Asturcón gave five haplotypes (from H_16 to H_20) which were not present in the black-coated Asturcón. In both Asturcón strains H_1 was the most frequent haplotype (50% and 30% in bay-coated and black-coated Asturcón, respectively). H_1 was identified in all the populations analysed. Three haplotypes identified in black-coated Asturcón were not identified in bay-coated Asturcón. The haplotypes shared between the two Asturcón pony strains gathered 76% (bay-coated) and 81% (black-coated) of the samples available in each Asturcón strain. Haplotypic and nucleotide diversity were slightly higher in bay-coated Asturcón pony (0.027 ± 0.006 and 0.021 ± 0.011, respectively) than in the bay-coated strain (0.024 ± 0.013 and 0.016 ± 0.009, respectively). The out-of-Sueve black-coated Asturcón population had relatively high haplotype diversity (0.091 ± 0.039) but particularly high nucleotide diversity (0.024 ± 0.013). Fig. 2 illustrates the high haplotypic diversity assessed. No clear phylogenies could be ascertained. Most sequences belonged to the haplotypic superfamily D (62% and 42% in bay-coated and black-coated Asturcón, respectively). In any case, both Asturcón strains shared sequences assigned to 10 of the haplogroups reported by Jansen *et al*. (2002) (see Table 1). AMOVA analyses did not give evidence of genetic structure. Regardless the model fitted, most genetic variability was assigned to the individuals, varying from 96.34% to 99.81%. When the analysis was fitted in two hierarchical levels, considering the population sampled or the strain, the Φ_{ST} values estimated were low and statistically non significant. The three hierarchical levels analysis, considering both the Asturcón strains and the populations within strain, gave a significant Φ_{CT} (relative divergence between strains) of 0.051, explaining 5.10% of the genetic variability. However, this may be an artifact due to the fact that between-populations differentiation within strains took a negative value. Further analyses (not shown) informed that Φ_{ST} distance computed between each pair of geographical populations were low and statistically non significant.

The current research points out the existence of noticeable haplotypic diversity in the sampled Asturcón pony populations. Despite the intense genetic bottleneck underwent by the Asturian native pony populations, a considerable number of matrilines remained. Domestic horses originated from multiple matrilines (Vilà *et al*., 2001; Jansen *et al*., 2002). However, it has also been reported that genetic bottlenecks, such as that well known occurred in the Exmoor pony, do not always affect haplotype diversity (Jansen *et al*., 2002). In any case, the analysed sequences were obtained considering founder matrilines only. This is clearly biasing upwards the overall diversity of the populations studied which is probably lower in the whole present population (Álvarez *et al*., 2012).

The analyses carried out also failed in assessing any statistically differentiation among Asturcón geographical populations or strains. Since the Asturcón populations analysed have been reproductively isolated for decades, one could expect some genetic differentiation due to genetic drift. Transmission of mtDNA is subject to major stochastic processes, such as practically inexistent paternal transmission, which can be amplified by local bottlenecks (White *et al*., 2008). Many different local processes of drift and fixation could have affected the maternal genetic scenario in the Asturcón pony. However, if happened, they could not be traced using the current data.

Furthermore, mitochondrial lineages have been widely shown as not useful to identify horse breeds. From a technical point of view, it is difficult to establish a pattern for horse mtDNA haplotypes neither at a regional nor breed level: the ability of the species for move and the use of the horse as the primary means of long distance transportation leaded to extensive mixing

Table 1. Frequencies by geographical population and totals for the 16 mitochondrial DNA haplotypes identified, including 33 variable sites, in Asturcón pony. Sample size per geographical population is in brackets. Variable sites and GenBank accession numbers are also given. Vertical numbers indicate the variable site position according to the reference sequence. The following parameters characterising genetic variability of data were computed using the program Arlequin 3.5 (Excoffier & Lischer, 2010): number of haplotypes identified (*k*), haplotype (gene) diversity (*h*) and nucleotide diversity (π)

Haplotype	Group[a]	GenBank Acc. No.	Variable sites	Bay-coated strain (42)			Black-coated strain (37)		
			1111111111111111111111111111111111111 5555555555555555555555555555555555555 4455555555566666666666677777778888888 9923348899000012345560024770112233 4614624567123476590963960166016788	El Aguión (9)	Carondio (13)	La Bobia (20)	Sueve (26)	Out-of-Sueve (11)	Total
Hap_1	D_1	JQ229813	CCGTTCCGAGTTCGTACGATGTAGATTCACAAC	3	7	11	9	2	32
Hap_2	D_3	JQ229814	.......A.....A.....	1				1	2
Hap_3	D_1	JQ229815	.....C.....				2		2
Hap_4	G	JQ229816	T.AC...A....TA...A...C.AG....T...				3		3
Hap_5	D_2	JQ229817	.......A...C.....	2		1		2	5
Hap_6	A_3	JQ229818	T.AC.....CT....AG.A....C....G..		2	1	4	1	8
Hap_7	C_2	JQ229819	T.AC...A..C.T....A.....T...GT	1		1	7	1	10
Hap_8	A_1	JQ229820	T.AC.T.AG...T...TAG.AC...C.....		1		1	1	3
Hap_9	A_3	JQ229821	T.AC..TA...CT..G.AG.A....C..G.G..					1	1
Hap_10	A_1	JQ229822	T.AC.T..G...T...TAG.AC...C.....		1			1	2
Hap_11	C_1	JQ229823	T.AC...A....T.C..A.C.....T...G.					1	1
Hap_16	A_1	KC527020	T.AC.T.A....T...TAG.AC...C.....			2			2
Hap_17	—	KC527021	T.ACC..A.A..TA...A.....C.....			1			1
Hap_18	A_5	KC527022	TTAC.....CT....A....G..C.....	1	1	3			5
Hap_19	D_1	KC527023	.......A.....		1				1
Hap_20	A_7	KC527024	T.AC.T..G...T....AG.A.....	1					1
k				6	6	7	6	9	16
h				0.111	0.077	0.050	0.039	0.091	0.013
$sd_{(h)}$				0.052	0.030	0.016	0.011	0.039	0.002
π				0.016	0.017	0.016	0.02	0.024	0.019
sd(π)				0.010	0.010	0.009	0.011	0.013	0.010

[a] Haplogroups defined by Jansen *et al.* (2002); Hap_17 could not be assigned to any haplogroup with certainty.

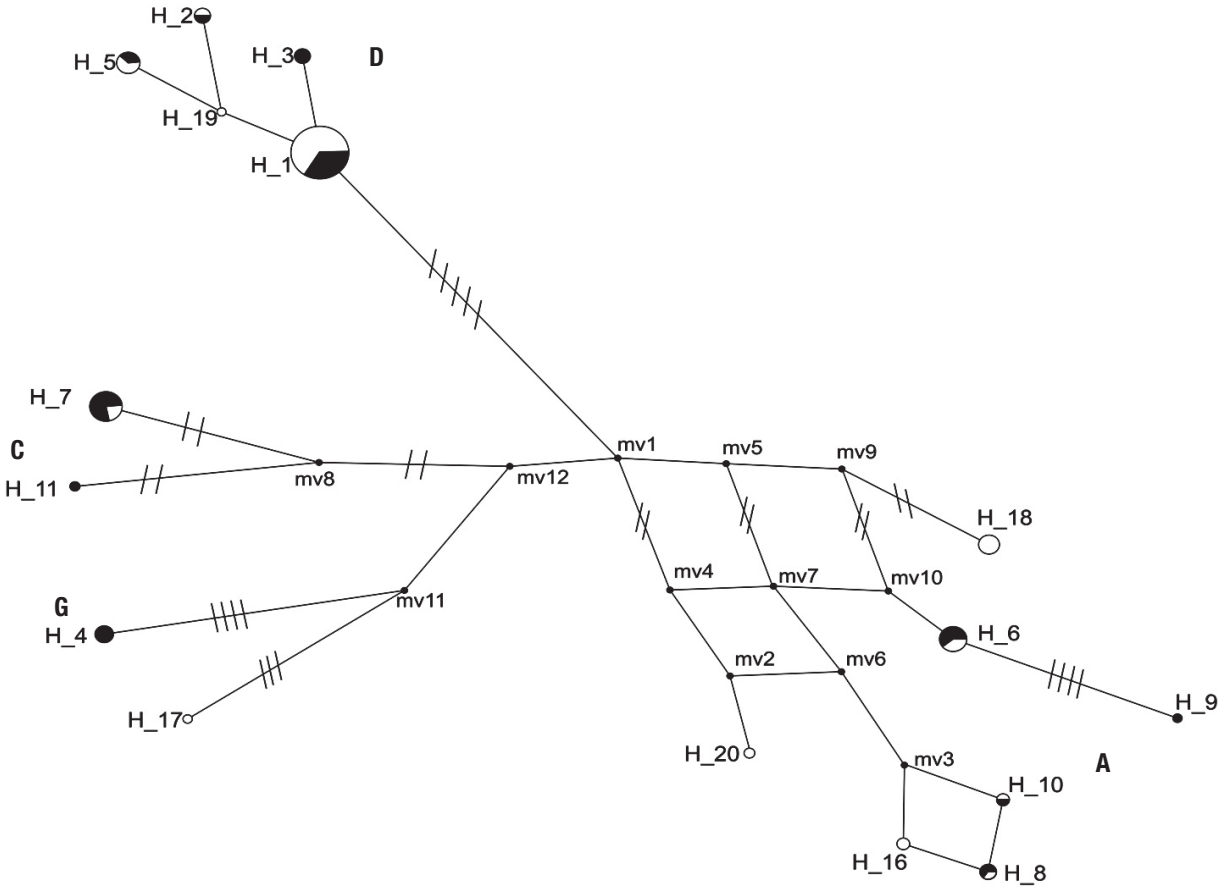

Figure 2. Median-joining tree constructed with the mtDNA haplotypes described in Table 1. Circles are proportional to the number of samples displaying each haplotype. Slashes on the branches mean the number of mutations (higher than 1) separating nodes. Capital letters near circles identify the haploclusters reported by Jansen *et al.* (2002) for the species. Within each circle, white portion represents the bay-coated Asturcón and black portion the black-coated Asturcón pony samples in the nodes. Missing haplotypes are denoted as mv.

of formerly distinct geographic populations before and after domestication (Vilà *et al*., 2001; Jansen *et al*., 2002). Recently, Achilli *et al*. (2012), using 83 horse mitochondrial genomes, suggested that the proposed specificity of certain mtDNA clusters to some horse breeds, such as the British pony breeds (Jansen *et al*., 2002), was only due to the traditional use of a small fragment of the control region in horse mtDNA studies. The information provided by the complete mitochondrial genome suggests that this specificity is no longer justifiable (Achilli *et al*., 2012). Moreover, during the last two centuries allochtonous horses have been exploited in the Asturian mountains. However, even though an influence of such breeds on the mitochondrial DNA composition of the Asturcón pony cannot be excluded, it has been reported that these breeds (namely the so called Hispano-Bretón) are more likely present-day reservoirs of ancestral mtDNA lineages of Celtic ponies of the Iberian Peninsula (Pérez-Gutiérrez *et al*., 2008). Therefore, to trace any mtDNA influence on the maternal composition of the founder Asturcón mare populations analysed is unlikely.

Finally, the current research suggests that the two strains of the Asturcón pony breed derive from the same ancestral mare population. Most sequences analysed could be assigned to the Jansen *et al*. (2002) D haplofamily which has been suggested to be the main representative of the ancestral Iberian horse population (Royo *et al*., 2005a). This is also truth for the out-of-Sueve population of the black-coated Asturcón pony, which was obtained in areas with not many historical references on the existence of ancient local Asturcón herds. Even though this population gathers a relatively large number of different Jansen *et al*.'s (2002) haplogroups, the maternal composition of this, or any other population analysed, does not allow to consider it as a

representative of "other" different breed but to the Asturcón.

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References

Achilli A, Olivieri A, Soares P, Lancioni H, Kashani BH, Perego UA, Nergadze SG, Carossa V, Santagostino M, Capomaccio S *et al.,* 2012. Mitochondrial genomes from modern horses reveal the major haplogroups that underwent domestication. P Natl Acad Sci USA 109: 2449-2454.

Álvarez I, Royo LJ, Pérez-Pardal L, Fernández I, Lorenzo L, Goyache F, 2011. Assessing diversity losses due to selection for coat colour in the endangered bay-Asturcón pony using microsatellites. Livest Sci 135: 199-204.

Álvarez I, Fernández I, Lorenzo L, Payeras L, Cuervo M, Goyache F, 2012. Founder and present maternal diversity in two endangered Spanish horse breeds assessed via pedigree and mitochondrial DNA information. J Anim Breed Genet 129: 271-279.

Álvarez-Llana J, 1995. Morfología y caracteres raciales. In: Asturcones (Álvarez-Llana J, Álvarez-Sevilla A, Jáuregui-Campos J, eds). Ediciones Caja de Asturias, Oviedo, Spain. pp: 3-39.

Álvarez-Sevilla A, 1995. Pasado y presente de la raza. In: Asturcones (Álvarez-Llana J, Álvarez-Sevilla A, Jáuregui-Campos J, eds). Ediciones Caja de Asturias, Oviedo, Spain. pp: 145-214.

Bandelt H-J, Forster P, Rohl A, 1999. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16: 37-48.

Excoffier L, Lischer HEL, 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10: 564-567.

García-Dory MA, 1980. Asturcón. Caballo de los astures. Ediciones Caja de Ahorros de Asturias, Oviedo, Spain. 84 pp.

Goyache F, Álvarez I, Fernández I, Pérez-Pardal L, Royo LJ, Lorenzo L, 2011. Usefulness of molecular-based methods for estimating effective population size in livestock assessed using data from the endangered black-coated Asturcón pony. J Anim Sci 89: 1251-1259.

Jansen TP, Forster MA, Levine H, Oelke M, Hurles M, Renfrew C, Weber J, Olek K, 2002. Mitochondrial DNA and the origins of the domestic horse. P Natl Acad Sci USA 99: 10905-10910.

Pérez-Gutiérrez LM, De la Peña A, Arana P, 2008. Genetic analysis of the Hispano-Breton heavy horse. Anim Genet 39: 506-514.

Royo LJ, Álvarez I, Beja-Pereira A, Molina A, Fernández I, Jordana J, Gómez E, Gutiérrez JP, Goyache F, 2005a. The origins of Iberian horses assessed via mitochondrial DNA. J Hered 96: 663-669.

Royo LJ, Álvarez I, Fernández I, Gutiérrez JP, Gómez E, Goyache F, 2005b. Characterisation of the founder matrilines in Asturcón pony via mitochondrial DNA. Proc 56th Annual Meeting of the European Association of Animal Production no 11, Uppsala (Sweden), 5-8 June. p: 98.

Royo LJ, Álvarez I, Gutiérrez JP, Fernández I, Goyache F, 2007. Genetic variability in the endangered Asturcón pony assessed using genealogical and molecular information. Livest Sci 107: 162-169.

Sambrook J, Fritsch EF, Maniatis T, 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. 545 pp.

Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599.

Vilà C, Leonard JA, Götherstoöm A, Marklund S, Sandberg K, Lindén K, Wayne RK, Ellegren H, 2001. Widespread origin of domestic horse lineages. Science 291: 474-477.

White DJ, Wolff JN, Pierson M, Gemmell NJ, 2008. Revealing the hidden complexities of mtDNA inheritance. Mol Ecol 17: 4925-4942.

Xu X, Arnason U, 1994. The complete mitochondrial DNA sequence of the horse, *Equus caballus*: extensive heteroplasmy of the control region. Gene 148: 357-362.