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Iberian Origins of New World Horse Breeds

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Abstract

Fossil records, archaeological proofs, and historical documents report that horses persisted continuously in the Iberian Peninsula since the Pleistocene and were taken to the American continent (New World) in the 15th century. To investigate the variation within the mitochondrial DNA (mtDNA) control region of Iberian and New World horse breeds, to analyze their relationships, and to test the historical origin of New World horses, a total of 153 samples, representing 30 Iberian and New World breeds, were analyzed by sequencing mtDNA control region fragments. Fifty-four haplotypes were found and assigned to seven haplogroups. Reduced levels of variation found for the Menorquina, Sorraia, and Sulphur Mustang breeds are consistent with experienced bottlenecks or limited number of founders. For all diversity indices, Iberian breeds showed higher diversity values than South American and North American breeds. Although, the results show that the Iberian and New World breeds stem from multiple origins, we present a set of genetic data revealing a high frequency of Iberian haplotypes in New World breeds, which is consistent with historical documentation.

The genus *Equus* evolved in North America, and during the first major glaciations of the late Pliocene (2–3 million years ago), some species crossed to Eurasia. North American and South American *Equus* species became extinct about 10,000 years ago from causes that are not fully understood, probably due to a combination of overhunting by humans and environmental causes, such as climatic changes (e.g., Clutton-Brock 1996).

However, horses persisted continuously on the Iberian Peninsula since the Pleistocene (1.8 million years ago) even during the Mesolithic, when the horse became extinct north of the Pyrenees (Gonzaga 2004).

Gene flow between horse populations of Iberia and North Africa occurred at multiple times throughout history. Exchanges between these two regions were particularly frequent during the long period of occupation of the Peninsula by the Moors (AD 711–1492), who brought Barb horses from North Africa (e.g., Oom 1992).

Horses only returned to the American continent (the New World) in 1493, with the navigator Christopher Columbus and during the subsequent Spanish colonization period (Bort 2004; Primo 2004). Those stallions and mares were bought in Seville's province, mainly from the peasant stock bred in the islands and salt marshes of the Guadalquivir River (Bort 2004).

Historical records report the presence of around 70 horses on the first colony of La Española (Dominican Republic and Haiti) by the year 1503. Subsequently, horses were brought into Panama (1514), Mexico (1524), Brazil (1531), Peru (1532), Argentina (1535), and Florida (1538) (Digard 1994). By 1553, there were some 10,000 free-roaming horses in the area of Queretaro (Mexico) that spread throughout North and South America (Clutton-Brock 1992).

Analysis of the mitochondrial DNA (mtDNA) control region sequence diversity has been an important tool for understanding the origin and diversification of domestic horses. Studies by Jansen et al. (2002), Lister et al. (1998), and Vilà et al. (2001) all point to extensive variation within and among breeds, with little congruence of haplotype assignment to breed or geographic region. These studies suggest that the domestic horse arose from several distinct wild horse populations and distributed over a moderately extensive geographic region large enough to contain considerable preexisting haplotype diversity and that there was considerable mixing of these haplotypes after domestication.

Here we compare the mtDNA control region variation in Iberian and New World horse breeds in order to understand their relationships and test the accuracy of historical documentation of New World horse origins.

Table 1. Number of samples sequenced in this study and gathered from GenBank and the respective main geographic location, breed, breed's code, and GenBank accession numbers

Geographic location	Breed	Code	Number of samples		Accession number
			This study	GenBank	
Iberia	Asturcon	AST	—	6	AY519872, AY519875–76, AY519879–81
	Caballo de Corro	CCO	—	6	AY519884–86, AY519888, AY519890, AY519897–98
	Cartujano	CTJ	—	6	AY519897–98, AY519900, AY519902, AY519904, AY519906
	Garrano	GR	3	3	**AF516500–01, AY997193,** AY246231, AY246233, AY246235
	Lusitano	LUS	6	—	**AF516502–05, AY997194–95**
	Losino	LOS	—	6	AY519924–25, AY519928, AY519930, AY519932, AF466009
	Mallorquina	MA	—	2	AF466013–14
	Marismeño	MAR	—	6	AY519934, AY519936, AY519938, AY519940, AY519942, AY519944
	Menorquina	ME	—	2	AF466015–16
	Potoka	POT	—	6	AY519958–60, AY519963, AY519967, AF4666012
	Pura Raza Española	PRE	6	—	**AY917165–66, AY917168, AF516509–11**
	Sorraia	SOR	—	2	AF447764–65
	Barb	BA	—	6	AJ413658, AJ413661, AJ413664–66, AJ413668
North America	Florida Cracker	FC	3	—	**AY997150–51, AY997192**
	Kiger Mustang	KM	6	—	**AF516489–90, AY997152–55**
	Spanish Mustang	SM	4	—	**AY997178–81**
	Sulphur Mustang	SUL	6	—	**AF516494–95, AY997187, AY997200–02**
	Mustang	MU	—	6	AJ413753, AJ413797, AJ413802, AJ413804, AJ413807, AJ413817
South America	Argentine Criollo	AC	1	5	AF465986–90, **AY997128**
	Brazilian Criollo	CR	6	—	**AF516496, AF516498, AY997145, AY997147, AY997149, AY997190**
	Campolina	CMP	6	—	**AY997139–44**
	Chilean Criollo	CC	4	—	**AY997131–34**
	Chilote	CH	4	—	**AY997135–38**
	Mangalarga	BM	5	—	**AF516506–08, AY997129–30**
	Mangalarga Marchador	MM	4	—	**AY997156–59**
	Pantaneiro	PN	6	—	**AY997160–64, AY997199**
	Paso Fino	PF	6	—	**AF516491–93, AY997197–99**
	Peruvian Paso Fino	PVP	5	1	**AF465993, AY997169–73**
	Puerto Rican Paso Fino	RP	4	—	**AY997174–77**
	Venezuelan Spanish	VS	5	—	**AY997182–86**

The accession numbers in bold are new ones generated by this study.

Materials and Methods

DNA Samples

We analyzed 90 samples, representing 3 Iberian, 4 North American, and 12 South American breeds (Table 1). When pedigree information was available, we selected unrelated individuals.

DNA was extracted from (1) fresh whole-blood samples after a high-salt extraction procedure (Montgomery and Sise 1990), (2) frozen whole-blood samples using the QIAmp Mini Blood Kit (Quiagen Inc., Valencia, CA), (3) frozen blood lysates using the Puregene Genomic DNA Isolation Kit (Gentra Systems Inc., Minneapolis, MN), and (iv) hair roots using Chelex-100 (Walsh et al. 1991).

DNA Sequencing

The universal primers L15926 and H16498 (Kocher et al. 1989) were used to amplify 360- to 442-bp fragments between sites 15411 and 15852 according to the horse reference sequence X79547 (Xu and Árnason 1994), including the

$tRNA^{pro}$ and hyper variable region I of the mtDNA control region.

The polymerase chain reaction (PCR) cycle sequencing reactions were performed on both strands, twice for each sample, using the CycleReader™ Auto DNA Sequencing Kit (MBI Fermentas GmbH, St. Leon-Rot, Germany), with infrared dye 800–labeled PCR primers used as sequencing primers and with the ABI™ Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Inc., Boston, MA). Sequences were determined with a Li-Cor™ 4200S Sequencer and an ABI 377 DNA Sequencer and were analyzed, respectively, with Li-Cor Image Analysis™ software and Sequencing Analysis Software™ v3.4.1 with free Factura™.

The sequences obtained in this study were deposited in GenBank database, and their accession numbers are shown in Table 1.

We also included in this study mtDNA control region sequences available from GenBank for 14 other Iberian and New World horse breeds (see Table 1). The Barb horse sequences were included as part of the Iberian group due to the historical link between this breed and the Iberian breeds.

Sequences were aligned using the CLUSTALW software (Thompson et al. 1994) and truncated to 288 bp, between positions 15483 and 15770, according to the horse reference sample X75947 (Xu and Árnason 1994), allowing the comparison with published sequences.

Data Analysis

Nucleotide diversity and haplotype (gene) diversity were obtained with the DNASP 4.00.5 software (Rozas et al. 2003), while the mean number of pairwise differences (MNPD) was obtained using the ARLEQUIN 2000 software (Schneider et al. 2000).

Median-joining network (Bandelt et al. 1999) was generated using NETWORK 4.1.0.8 software (available at http://www.fluxus-engineering.com).

Results

mtDNA Lineages in Iberian and New World Horse Breeds

We sequenced 90 individuals and identified 26 haplotypes of which 10 were new, namely, Hap_36, Hap_37, Hap_39, Hap_42, Hap_43, Hap_44, Hap_45, Hap_47, Hap_49, and Hap_50 (accession numbers AY997128, AF516507, AY997138, AY997131, AF516503, AY997158, AY997159, AF516492, AY997176, and AY997177, respectively). These haplotypes belonged to one Iberian and seven South American breeds (Table 1, Figure 1).

Our analysis was based on a sample set composed of 90 sequences from this study and 63 available from GenBank. The 153 mtDNA control region sequences yielded 54 different haplotypes defined by 44 polymorphic sites: 43 transitions and 1 transversion. For each breed, we identified from two to six haplotypes, differing from the reference sequence (GenBank X79547) by 1–11 sites within the 288 bp analyzed (the table with all this information is available from the authors on request).

Fifteen haplotypes (27.8%) were detected more than once, and 39 (72.2%) were singletons: 21 (53.8%) from Iberia (corresponding to 10 breeds), 2 (5.1%) from North America (one breed), and 16 (41%) from South America (eight breeds). Potoka is the breed with the highest percentage of singletons (100%) followed by Puerto Rican Paso Fino and Argentine Criollo, with 75% and 67%, respectively.

The 54 haplotypes (26 from the present study) could be assigned to five (D1, D2, D3, C2, and A4) out of the 17 major mtDNA lineages defined by Jansen et al. (2002). Cluster D1 is considered, by these authors, as representative of Iberian and North African breeds.

We further analyzed our sequences, and according to the presence of specific point mutations, we defined a total of seven major haplogroups. Their names indicate the mutation position and nucleotide used as diagnostic of the haplogroups. As these point mutations were also present in clusters defined by Jansen et al. (2002), we decided to incorporate the corresponding letters of these clusters into the haplogroup names. The sequences were assigned to the haplogroups, and results are presented in Table 2.

Figure 1 shows a median-joining network relating the mtDNA sequences of the analyzed breeds and the haplogroups defined here.

Haplogroup $D_{494C,496G,534T,603C,649G}$ is composed of individuals from all breeds (Iberia, 44%; North America, 60%; South America, 48%) except from Menorquina and Sorraia. Included in this haplogroup is the modal sequence Hap_1 (D1 from Jansen et al. 2002) seen in high frequency in the Iberian and New World horse breeds analyzed here and having the highest overall frequency in all the geographic regions (Iberia, 26%; North America, 44%; South America, 29%).

Haplogroup $A_{542C,666A}$ was the second most common with higher frequency of sequences in our sample set (12.4%) being found in all geographic regions (Iberia, 10.6%; North America, 24%; South America, 9.7%).

Haplogroup $A_{542T,666A}$ almost exclusively comprised Iberian breeds, the exception was the North American Sulphur Mustang breed. Haplogroup C_{617C} comprised only South American breeds, and C_{601C} was predominantly represented by breeds from that region.

Diversity of Iberian and New World Breeds

Results from MNPD, nucleotide diversity, and haplotype diversity showed a similar pattern in all breeds (Table 3). The Sulphur Mustang presented the lowest values and Mallorquina the highest for all diversity indices, with the exception of haplotype diversity where several breeds had the maximum value. For all diversity indices, Iberian breeds showed higher diversity values followed by South American and North American breeds (Table 4).

Discussion

From the 12 Iberian breeds studied, we provide new information for three: Garrano, Lusitano, and Pura Raza Española. Among the Iberian breeds, Menorquina and Sorraia showed

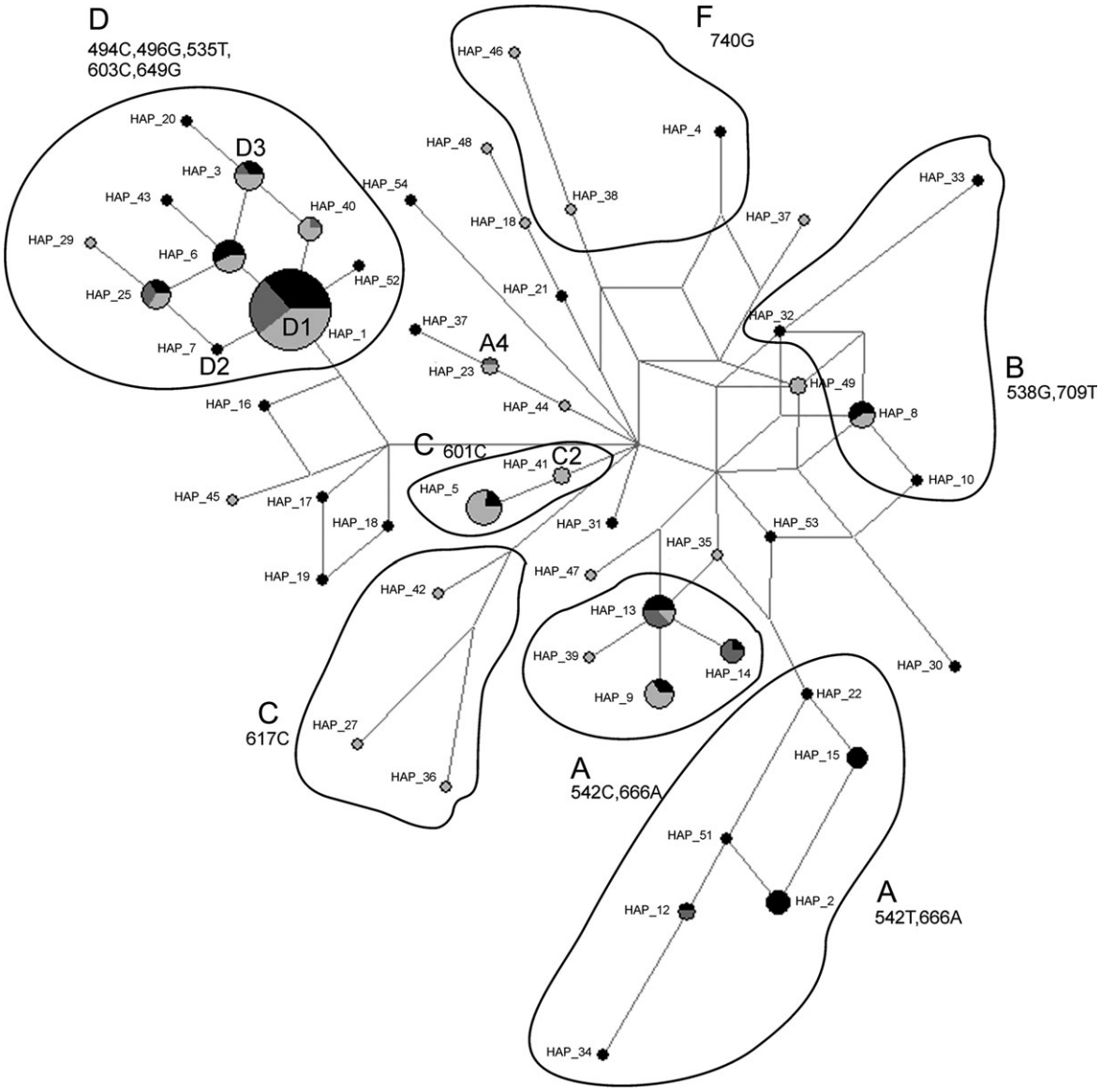

Figure 1. Median-joining network relating the mitochondrial DNA D-loop sequences observed in Iberian and New World horse breeds. Black represents Iberian Peninsula individuals, dark gray represents North American individuals, and light gray represents South America individuals. Circle size is proportional to sequence frequency. Free-form shapes represent haplogroups defined by certain point mutations. D1, D2, D3, C2, and A4 are clusters previously defined by Jansen et al. (2002). See Table 2 for haplogroup characteristics.

the lowest diversity values. Menorquina (from an island of Spain with the same name) was established as a breed in the early 1980s (Gallardo PP and Andres Cara DF, unpublished). Approximately 70 horses were selected based on phenotypic characteristics and, since 1994, have been bred in a closed system. At the time of this breed's establishment, a bottleneck effect surely occurred; however, we cannot exclude the sample size as a cause for this low diversity value. For the Sorraia horse, the lower diversity found was in accordance with previous studies using different genetic markers, namely, blood groups and biochemical polymorphisms (Oom and Cothran 1994), mtDNA (Luís et al. 2002a), microsatellites (Luís et al. 2002b), and major histocompatibility complex genes (Luís et al. 2005). This Portuguese horse breed was recovered in 1937 from 12 founders (five males and seven females), and only two maternal lineages are presently represented in the living population (Luís et al. 2002a).

Of the five North American breeds analyzed, the Florida Cracker, Spanish Mustang, and Sulphur Mustang were tested for the first time. The North American samples showed lower numbers of haplotypes and haplotype diversity compared to the Iberian and South American breeds. All the North American populations in this analysis represent horses derived from feral populations. The Kiger, Florida Cracker,

Table 2. Characterization of the haplogroups defined in this study with indication of mutation points, diagnostic nucleotide, and percentage of total samples from each geographic region assigned to each haplogroup

Haplogroup name	Mutation points	Diagnostic nucleotide	Percentage of total samples in each haplogroup (%)			Jansen et al. (2002) lineages
			Iberia	South America	North America	
$A_{542T,666A}$	15542	T	12	—	8	A1 and A2
	15666	A				
$A_{542C,666A}$	15542	C	11	10	24	A3
	15666	A				
$B_{538G,709T}$	15538	G	12	3	—	B1 and B2
	15709	T				
C_{601C}	15601	C	1.5	15	—	**C2**
C_{617C}	15617	C	—	5	—	C1
$D_{494C,496G,534T,603C,649G}$	15494	C	44	48	60	**D1**, **D2**, and **D3**
	15496	G				
	15534	T				
	15603	C				
	15649	G				
F_{740G}	15740	G	1.5	3	—	F2
Others[a]	—	—	18	16	8	**A4**

We also indicate lineages defined by Jansen et al. (2002) that were screened (bold) and lineages that despite not being found in this sample set would belong to our defined haplogroups because they have the diagnostic point mutations.

[a] Haplotypes that are not assigned to the defined major haplogroups.

Sulphur Mustang, and Spanish Mustang groups have recently become represented by breed registries. The first three are from single, isolated populations in Oregon, Florida, and Utah, respectively. The Spanish Mustang breed was formed with horses that originated from feral or Native American stock from all over North America. All were selected based on a phenotype that was believed to represent Spanish ancestry. The Mustang group also is a collection of horses with feral origins and presumed Spanish physical characteristics. It is likely that the Kiger, Florida Cracker, and Sulphur Mustang breeds experienced bottlenecks or a limited number of founders, resulting in lower diversity. The Sulphur Mustang has the lowest diversity with only two haplotypes found among six individuals. The Spanish Mustang and Mustang groups have the highest diversity of the North American breeds, comparable to the higher values for the other groups, which reflects their more diverse origins.

From the 12 South American breeds considered in our study, 10—Brazilian Criollo, Campolina, Chilean Criollo, Chilote, Mangalarga, Mangalarga Marchador, Pantaneiro, Paso Fino, Puerto Rican Paso Fino, and Venezuelan Spanish—are analyzed for the first time. The higher variability of the South American breeds compared with the North American ones may be explained by the founding of North American breeds with horses coming from Mexico and the Caribbean. The South American breeds also have been selected for a greater diversity of forms and uses when compared to the North American horses, and some have been crossed to other non-Iberian type horses (Hendricks 1995).

In comparison with the New World breeds, the Iberian samples showed the highest values for the diversity parameters analyzed (including the frequency of singletons). This finding supports the historical documentation that Iberia was the source of much of the original stock that was used to populate the New World with horses. Also Iberia experienced an active interchange of horses with other breeding countries, such as the Pontic-Caspian steppes, Gaul, Italy, Macedonia, and Greece (Gonzaga 2004), that might have increased the variability of the Iberian horses. Therefore, the diversity in this region would be expectedly higher. The low variation in the New World breeds may be an indication of founder effect or a bottleneck during their establishment, an hypothesis previously suggested by Mirol et al. (2002) in their work with Argentinean Criollo.

The high diversity of the mtDNA control region within the studied horse breeds confirms a differentiated ancestry, previously indicated by several authors (e.g., Hill et al. 2002; Jansen et al. 2002; Keyser-Tracqui et al. 2005; Kim et al. 1999; Lister et al. 1998; Lopes et al. 2005; Mirol et al. 2002; Vilà et al. 2001). However, some haplotypes have been identified as corresponding to specific breeds/geographic areas, namely, D1, first identified by Jansen et al. (2002), and further emphasized by Lopes et al. (2005), as being well represented in Iberian breeds. Haplotype Hap_1, from our work, corresponds to D1 and was found in high frequency not only in the Iberian breeds but also in the New World ones. Besides Hap_1, the high frequency of Iberian and New World samples belonging to haplogroup $D_{494C,496G,534T,603C,649G}$ (48%) is striking. This haplogroup has been considered representative of the ancestral Iberian horse population (Royo et al. 2005). These two findings support the documented role of the Iberian breeds in the origin of New World horse populations.

The second haplogroup with more representatives of Iberian and New World horse breeds is $A_{542C,666A}$. This haplogroup includes Marismeño horses (stripped horses from

Table 3. Diversity indices in the analyzed breeds

Geographic location	Breed	n	nh	MNPD	SE	Nucl. diver.	SE	Hd	SE
Iberia	Asturcon	6	4	6.67	3.67	0.023	0.015	0.800	0.172
	Caballo de Corro	6	3	3.80	2.22	0.013	0.009	0.733	0.155
	Cartujano	6	4	7.73	4.20	0.027	0.017	0.800	0.172
	Garrano	6	4	6.27	3.47	0.022	0.014	0.867	0.129
	Lusitano	6	6	7.80	4.24	0.027	0.017	1.000	0.096
	Losino	6	4	3.93	2.29	0.014	0.009	0.800	0.172
	Mallorquina	2	2	9.00	6.71	0.031	0.033	1.000	0.500
	Marismeño	6	4	6.20	3.43	0.021	0.014	0.867	0.129
	Menorquina	2	2	3.00	2.45	0.010	0.012	1.000	0.500
	Potoka	6	6	7.00	3.83	0.024	0.015	1.000	0.096
	Pura Raza Española	6	5	5.67	3.16	0.020	0.013	0.933	0.122
	Sorraia	2	2	3.00	2.45	0.104	0.012	1.000	0.500
	Barb	6	5	6.53	3.60	0.023	0.014	0.933	0.122
South America	Argentine Criollo	6	6	7.33	4.00	0.026	0.016	1.000	0.096
	Brazilian Criollo	6	4	3.20	1.92	0.011	0.008	0.867	0.129
	Campolina	6	4	4.33	2.49	0.015	0.010	0.867	0.129
	Chilean Criollo	4	4	5.50	3.34	0.019	0.014	1.000	0.177
	Chilote	4	3	5.50	3.34	0.019	0.014	0.833	0.222
	Mangalarga	5	5	6.60	3.76	0.023	0.015	1.000	0.126
	Mangalarga Marchador	4	4	5.50	3.34	0.019	0.014	1.000	0.177
	Pantaneiro	6	5	5.40	3.03	0.019	0.012	0.933	0.122
	Paso Fino	6	3	5.33	3.00	0.019	0.012	0.600	0.215
	Peruvian Paso Fino	6	3	4.40	2.53	0.015	0.010	0.600	0.215
	Puerto Rican Paso Fino	4	4	5.83	3.53	0.020	0.015	1.000	0.177
	Venezuelan Spanish	5	3	5.20	3.03	0.018	0.012	0.700	0.218
North America	Florida Cracker	3	2	5.33	3.53	0.019	0.015	0.667	0.314
	Kiger Mustang	6	3	3.27	1.95	0.011	0.008	0.733	0.155
	Spanish Mustang	4	3	7.00	4.17	0.024	0.017	0.833	0.222
	Sulphur Mustang	6	2	0.33	0.38	0.001	0.002	0.333	0.215
	Mustang	6	5	6.47	3.57	0.023	0.015	0.933	0.122

n, number of individuals; nh, number of haplotypes found; MNPD, mean number of pairwise differences; Nucl. diver., nucleotide diversity; Hd, haplotype (gene) diversity.

the Guadalquivir salt marshes) that, like the Sorraia, are considered a primitive Iberian equine type (Andrade 1954; Bort 2004) and therefore might have been extensively used for breeding in Iberia. The high frequency of New World horses in this haplogroup may be explained by historical records stating that mares taken to the American continent by Christopher Columbus and during the subsequent Spanish colonization period were bought mainly from the stock bred in the islands and salt marshes of the Guadalquivir River (Bort 2004).

Of the three geographic regions studied, South America is the only one having sequences that belong to haplogroup C_{617C}, and it is also the region having almost the exclusive representation in haplogroup C_{601C}. Indeed, the only non–South American samples that belong to this latter haplogroup are two individuals from the Caballo de Corro breed, a Celtic origin pony from Asturias. These two haplogroups named "C" share some mutation points with cluster C1 from Jansen et al. (2002), who consider this as distinctive for northern European ponies, known to have Celtic origin. These findings may indicate common matrilineal ancestors between Celtic ponies and South American breeds, a result that is in accordance with historical records because in 1508 the Spanish crown authorized the transport of 40 Celtic type horses (small and resistant) in the expedition organized by Alonso Ojeda and Diego Nicuesa to Panama (Mirol et al. 2002).

The sharing of haplotypes between Iberia and New World, and especially of those belonging to the haplogroup considered as representative of the ancestral Iberian horse

Table 4. Diversity indices in the analyzed geographic regions

Geographic location	Total	nh	MNPD	SE	Nucl. diver.	SE	Hd	SE
Iberia	66	34	6.67	3.19	0.023	0.012	0.924	0.025
South America	62	27	5.52	2.69	0.019	0.010	0.898	0.030
North America	25	10	5.00	2.52	0.018	0.010	0.793	0.075

Total, number of individuals; nh, number of haplotypes found; MNPD, mean number of pairwise differences; Nucl. diver., nucleotide diversity; Hd, haplotype (gene) diversity.

population ($D_{494C,496G,534T,603C,649G}$), supports the widely accepted view of Iberian ancestry in American livestock (e.g., Miretti et al. 2004; Primo 2004).

Although extensive migrations in the past make it difficult to find clear connections between mtDNA haplotypes and geographic groups, we present a set of genetic data revealing that New World breeds have a high frequency of haplotypes of Iberian origin and represent a subset of the diversity found in Iberia. Therefore, this study supports the historically documented Iberian origins of New World horses.

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