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ABSTRACT

Marismeño horses originated from primitive horses living around the marshes of the Guadalquivir River in Southern Spain. Throughout their evolution, they have experienced crosses with other breeds, first with horses from North Africa and thereafter with other horses. However, they have not lost their ability to adapt to the demanding marsh environment. Recently, a studbook of the breed was established, and the Breeders Association started a conservation programme. To study the relationship of the Marismeño with other breeds, a microsatellite analysis was developed, which included other ancient Southern Iberian horse populations, such as the Sorraia and Retuertas breeds. Candidates of recent crossbreeding with Marismeño horses, such as the Hispano-Arabian and the Spanish Purebred, were studied, and the Thoroughbred and the Arabian breed were used as international references. The results indicated that the Marismeño horse population maintains a great genetic diversity. Despite recent crossbreeding, the fixation index and the Hardy-Weinberg equilibrium analysis disclosed a certain homogeneity degree. A dendrogram was built using the obtained genetic distances, and clustering was performed with the software STRUCTURE, and the results reflected the genetic differentiation of the Marismeño horse from the other autochthonous Iberian breeds, although the Marismeño population has maintained a tight relationship with the Spanish Purebred. Remarkably, some relatedness between the Marismeño and the Barb horse breeds could be observed and was most likely derived from an ancient gene flow between the horses of the Iberian Peninsula and North Africa.

Introduction

The biodiversity conservation of livestock remains an important concern. Despite worldwide efforts following the Convention on Biological Diversity (CBD) held in 1992, approximately one-third of all breeds continue to be threatened with extinction. Even after the approval by the Spanish Government of the Marismeño horse breed in 2008, this breed continued to be chaotically managed until 2012, which was the year the Studbook was created. According to the Association's data, the breed, using an open nucleus breeding scheme, includes less than 1000 dams and only 20 sires. These data place the breed in the Food and Agriculture Organisation of United Nations (FAO

2000) endangered populations list (http://dad.fao.org). The Marismeño horse is also classified by the Spanish government as an endangered breed because of its low census (Real Decreto 2129/2008); only 1226 individuals are registered in the studbook (MAGRAMA 2016).

Ancient Southern Iberian horses developed into the Sorraia, Retuertas and Marismeño equine breeds. It has historically been assumed that these horse populations share a common origin with Barb horses subsisting on the other side of the Gibraltar Strait (Sotillo & Serrano 1985), although this fact has not been absolutely confirmed by previous molecular marker studies (Royo et al. 2005; Luís et al. 2006).

The horses that dwell in the Guadalquivir marshes, including the Doñana National Park, have traditionally been handled by local farmers. These horses hold great sociocultural importance for the area, and in view of the ancestral and typical horse traditions related to cattle handling, they are considered a true ancestor of the American cowboy herd management (Muñoz-Bort 2004). Overall, the existence of the Marismeño horse is a deep-seated part of the local culture and traditions.

The Marismeño horse is an equine with a typical withers height ranging from 140 to 148 cm, a subconvex profile, a broad chest and fine members (Figure 1). It is especially appreciated for its ability to work and its characteristics of adaptation, rusticity, disease resistance and courage; hence, it is very useful for traditional fieldwork. It is also important that the Marismeño horses that live in the Doñana National Park are sustained on food from the environment and that they do not receive any additional food, such as additional forage feed or fodder, or veterinary treatment (e.g., antiparasitary preventive treatments) because it is not allowed by the National Park guidelines. The distribution area of the Marismeño horse is very narrow and limited to the marshes of the Guadalquivir and mainly around the villages of Almonte and Hinojos located in the province of Huelva.

For decades, stallions of different breeds, mainly the Spanish Purebred and occasionally the Thoroughbred, were used to improve the height of the horses that live in the Guadalquivir marsh area (before the establishment of the Studbook in 2008) (Fernández Rodríguez 2010) to make them more appropriate for riding. Nonetheless, the severe marsh conditions prevented the survival of the non-native breeds, such as the Thoroughbred living in semi-feral conditions, and it was necessary to conduct crosses with well-adapted individuals to this harsh environment. This fact led to the production of a horse breed retaining many features of the first horses that populated this region combined with other characteristics, which make it more appropriate to take on current farming and riding actions.

DNA technology-based parentage control and breed assignments are very valuable tools for the control of a breed and its conservation. Microsatellite typing has been widely used in assorted domesticated animal diversity studies, including large international projects concerning goats (Nomura et al. 2012), chickens (Granevitze et al. 2007), sheep (Peter et al. 2007), pigs (Druml et al. 2012), horses (Vega-Pla et al. 2006; Conant et al. 2012) and cattle (Delgado et al. 2012).

The aim of this study was to analyse the diversity and population structure of the Marismeño breed using microsatellite markers. An additional seven horse breeds derived from the southern part of the Iberian Peninsula were included in the study to draw comparisons.

Materials and methods

Hair or blood samples were collected from randomly selected horses respective to each studied breed

Figure 1. Marismeño stallion in Guadalquivir Marshes.

(Table 1). Marismeños were obtained from 10 herds (one male and nine females per herd), and the rest of the breeds were randomly selected from our blood or hair sample bank. All of the Marismeño samples belonged to animals registered in the Foundational Studbook.

The DNA from hair samples was extracted by incubating 8–10 roots at 60° in 100 µl of 5% Chelex resin solution followed by 10 minutes of boiling, and the DNA from the blood samples was extracted following the method of Martínez et al. (2000).

Twenty-three microsatellite markers (Table 2) were amplified using the polymerase chain reaction (PCR) technique in a GeneAmp 9600 (Perkin Elmer, Norwalk, CT) by performing three multiplex reactions; 12 of them recommended for genetic characterisation by the FAO (2011) and 11 proposed by Tozaki et al. (2001). Sizing of PCR products was accomplished using

an internal size standard and a reference sample on each gel. The fragment analysis and allelic typing were performed using the software packages GeneScan Analysis 3.1.2 and Genotyper 2.5, respectively.

The average number of alleles per locus and the allelic richness index, corresponding to the minimum number of alleles based on the smallest sample size ($n = 21$), were estimated using the program FSTAT (Goudet 2001).

Allele frequencies, together with the observed (Ho) and expected (He) heterozygosities and the polymorphic information content (PIC), were calculated by the means of the Microsatellite TOOLKIT Add-In for Excel (Park 2001).

The F_{IS} values (the heterozygote-deficient coefficient as an estimation of the inbreeding coefficient across populations) with 95% confidence intervals were calculated with the GENETIX v. 4.04 software (Belkhir et al. 2004), whereas the Hardy–Weinberg equilibrium (HWE) was tested using the programme GENEPOP v. 3.1c (Raymond & Rousset 1995), which applies Fisher's exact test through the Markov chain Monte Carlo method (Guo & Thompson 1992).

The D_A genetic distance (Nei et al. 1983) was calculated using the software POPULATIONS 1.2.28 (Langella 1999). A neighbour-joining tree with the D_A genetic distances was built and then graphically represented with the software TreeView (Page 1996) to highlight the differentiation and potential associations among the diverse breeds.

Table 1. Sample size and herds (N/Herds), average number of alleles per population (ANA), observed average heterozygosity (Ho), expected average heterozygosity (He) and inbreeding coefficient (F_{IS}).

Breed	N/Herds	ANA	Ho	He	F_{IS}
Arabian	60/60	6.32	0.6547	0.6754	0.0307
Barb	21/4	7.32	0.7657	0.7598	−0.0080
Hispano-Arabian	40/15	7.24	0.7520	0.7664	0.0190
Spanish Purebred	60/60	7.64	0.7138	0.7388	0.0341
Thoroughbred	60/60	5.72	0.7175	0.7348	0.0236
Retuertas	67/1	6.76	0.7359	0.7137	−0.0313*
Sorraia	23/1	4.48	0.6194	0.6418	0.0357
Marismeño	50/10	8.48	0.8016	0.7938	−0.0099

*Significance level ($p < .05$).

Table 2. Microsatellites analysed, multiplex PCR conditions, number of alleles per marker (NA), allelic richness (RA), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC), inbreeding coefficient (F_{IS}) and p-value for the deviations from the Hardy–Weinberg equilibrium.

Microsatellite	Multiplex	NA	RA	Ho	He	PIC	F_{IS}	p-Value
AHT4	M1	7	6.859	0.7869	0.7901	0.7511	0.044	0.1596
AHT5	M1	6	5.861	0.7500	0.7876	0.7407	0.048	0.4454
ASB17	M1	11	9.982	0.7869	0.8820	0.8619	0.004	0.0757
ASB2	M2	12	9.000	0.8036	0.8740	0.8517	−0.071	0.9475
ASB23	M1	7	5.969	0.8596	0.8075	0.7704	−0.046	0.9659
HMS3	M2	8	7.000	0.7500	0.8174	0.7868	0.088	0.0418*
HMS6	M1	6	5.969	0.7193	0.7198	0.6769	0.026	0.1906
HMS7	M1	6	5.000	0.7541	0.7407	0.6977	−0.061	0.1353
HTG10	M2	8	5.999	0.7167	0.7230	0.6779	−0.021	0.3122
HTG4	M1	7	4.998	0.6721	0.7420	0.6925	0.077	0.0219*
LEX33	M2	11	8.000	0.8070	0.8536	0.8279	−0.045	0.3195
VHL20	M1	9	8.843	0.8056	0.8658	0.8363	0.071	0.1337
TKY287	M3	8	7.905	0.8136	0.7618	0.7235	−0.095	0.3859
TKY294	M3	8	6.987	0.7167	0.7443	0.7091	0.035	0.3068
TKY297	M3	9	7.884	0.7333	0.7908	0.7555	0.141	0.1474
TKY301	M3	7	6.980	0.8361	0.8090	0.7743	−0.038	0.4651
TKY312	M3	10	8.843	0.8852	0.8153	0.7874	−0.043	0.1416
TKY333	M3	8	6.000	0.9180	0.8302	0.7988	−0.087	0.6079
TKY337	M3	7	6.771	0.6571	0.7255	0.6798	0.095	0.0952
TKY341	M3	7	6.874	0.8333	0.7452	0.7025	−0.159	0.4428
TKY343	M3	10	9.943	0.8033	0.7843	0.7559	0.004	0.7333
TKY344	M3	8	7.716	0.7049	0.7513	0.7090	0.076	0.0347*
TKY394	M3	8	7.874	0.7500	0.8066	0.7740	0.115	0.0981
Average		8.24	7.272	0.7767	0.7899	0.7540	0.007	

*Significance level ($p < .05$).

The genetic structure of the breeds was analysed with the software STRUCTURE v 2.3.4 (Pritchard et al. 2000), which identifies clusters of related individuals from multilocus genotypes and assigns individuals to identified clusters using a Bayesian algorithm based on the Markov chain Monte Carlo method. The analysis involved an admixture model with correlated allele frequencies. Eight independent runs were conducted with 300,000 iterations during the burn-in phase and 1,000,000 iterations for sampling from $K = 2$ to $K = 9$ (K = number of clusters) to estimate the most likely number of clusters present in the dataset. The STRUCTURE results in graphic representations were obtained with the programme DISTRUCT 1.1 (Rosenberg 2004). Eight independent simulations from $K = 2$ to $K = 9$ were performed to identify the most likely K through ΔK modal's distribution determination (Evanno et al. 2005) using the program STRUCTURE HARVESTER (Earl & vonHoldt 2012). This test is used to detect the ability of the algorithm used by the program STRUCTURE to assign individuals to its previously known cluster of origin when there are more than 2 populations. This analysis is used to detect the number of genetic groups that fit better in the dataset using the ΔK modal's distribution determination. The outcome depends on the marker used, the number of loci analysed, the number of sampled populations and the number of individuals per sample. The genetic structure is not always a reflection of the geographical distance between the populations.

Results

All microsatellite markers showed high levels of polymorphism with an average of 8.24 alleles per locus (Table 2), evidencing a minimum of 6 alleles (AHT5, HMS6 and HMS7) and a maximum of up to 12 alleles (ASB2). This high-allelic diversity was also confirmed by the high allelic richness (7.36) value observed, which provides an idea of a breed's allelic diversity regardless of the number of samples analysed.

The highest expected heterozygosity was found for the ASB2 (0.87) marker, whereas the lowest was detected for the HMS6 (0.72) microsatellite.

The observed heterozygosity values ranged from 0.66 to 0.92. All of the markers showed PIC values higher than 0.50; therefore, all of the microsatellite markers screened were informative and detected genetic variability in all of the studied populations.

Table 2 shows that only three markers significantly deviated from the Hardy–Weinberg equilibrium. The observed average F_{IS} value of 0.007 indicated that no clear heterozygosity deficiencies or other excesses were observed.

The mean values of He and Ho are 0.79 and 0.74, respectively. A mean of 6.75 alleles per population was detected; the Sorraia and Thoroughbred populations showed the lowest number of alleles (4.48 and 5.72, respectively), and Marismeño showed the highest (8.48). The observed and expected heterozygosities in each breed are shown in Table 1. The Retuertas (RET) population showed a significant excess of heterozygosity and a low F_{IS} value. Negative F_{IS} values were also found in other breeds, but observed differences were not significant. Furthermore, only 13 significant deviations from the Hardy–Weinberg equilibrium were detected among the 184 population-locus combinations, which is precisely the number expected by chance at the 5% level. No population showed more than three markers deviating from the Hardy–Weinberg equilibrium (data not shown).

The average F-statistics and their 95% confidence intervals were obtained after bootstraps of 10,000 iterations over loci comprised of $F_{IS} = 0.0104$, $F_{IT} = 0.0989$ and $F_{ST} = 0.0895$.

The smallest Nei's (Da) genetic distances were between the Marismeño population and the Spanish Pure Breed (Table 3). This is not surprising because the horses from the Guadalquivir marshes have traditionally been crossbred with Spanish Pure breed stallions. The neighbour-joining tree is shown in Figure 2. The Sorraia breed is clearly differentiated and far from the rest of the breeds represented in the tree. Interestingly, the Retuertas population did not cluster with any of the European breeds and was in contrast to the Sorraia, which was probably due to its geographic isolation and common origin in the South of the Iberian Peninsula. One cluster grouped the

Table 3. Genetic distances among the eight populations studied based on Da distances of Nei et al. (1983).

BREED	Arabian	Barb	Hispano Arabian	Spanish Purebred	Thoroughbred	Retuertas	Sorraia	Marismeño
Arabian	0							
Barb	0.1869	0						
Hispano-Arabian	0.0819	0.1398	0					
Spanish Purebred	0.1728	0.1376	0.0695	0				
Thorougbred	0.1967	0.2631	0.1753	0.2035	0			
Retuertas	0.2118	0.2080	0.1486	0.1390	0.2772	0		
Sorraia	0.3864	0.3332	0.3392	0.3106	0.3984	0.2805	0	
Marismeño	0.1344	0.1180	0.0762	0.0661	0.1535	0.1161	0.3005	0

Hispano-Arabian and Arabian horse with the Thoroughbred, with a shared origin, the Arabian horse is an ancestor of many present breeds. The Marismeño horse has a very short branch; this is likely due to its recent definition process (which is actually ongoing) and has stopped the introduction of stallions from other breeds. We can see the influence of the Spanish Pure Breed, Arabic and Barb horses on its gene pool. This is the reason why the Marismeño horse is found in the centre of the D_A distance tree.

Clustering and assignment tests were performed on the entire data set with an increasing number of inferred clusters. The cluster results showing a plot of the sample individuals is depicted in Figure 3. Independent runs from $K=2$ to $K=9$ produced consistent results. $K=2$ indicated the presence of the following two very distinct clusters: one corresponded to the Thoroughbred and the Arabian horses, whereas the other included the remaining analysed equine breeds. From clusters $K=4$ to $K=9$, the grouping tests reflected the presence of a population structure associated with a 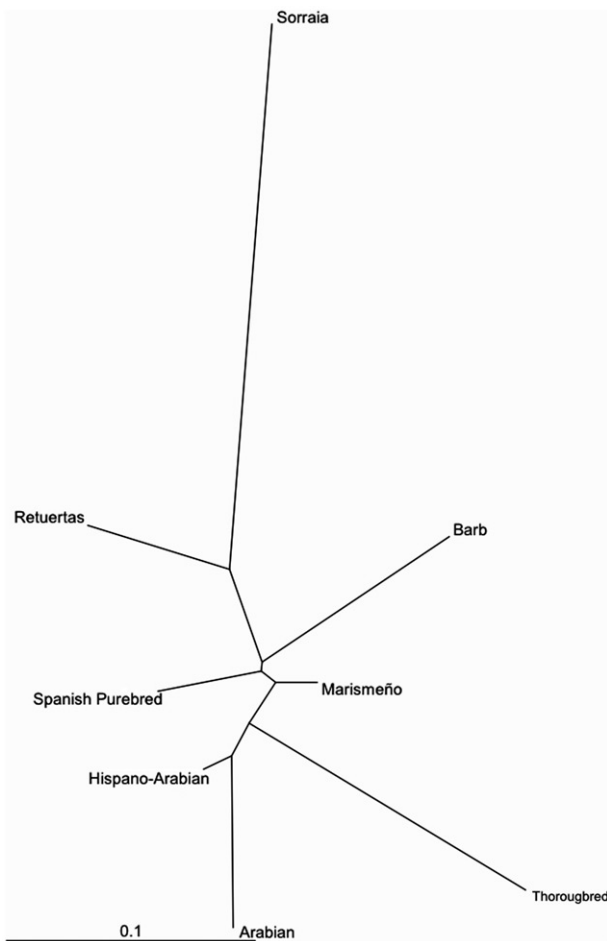progressive genetic differentiation of the autochthonous Iberian and North African breeds. Assignment tests isolated the Retuertas and Sorraia horses from the other breeds as early as $K=5$ and maintained their integrity thereafter. The Marismeño horse was clearly differentiated from its geographic neighbour, the Retuertas horse, starting from $K=4$, although both share the same origin.

The ΔK modal's distribution determination indicates that the soundest number of genetic groups fitting our data comprised $K=7$ as was evidenced in the lowest standard deviation and the location on top of the plateau once the real K value was reached. This finding completely coincides with the number of breeds typed considering that the Hispano-Arabian is a mixture of the Spanish Purebred with the Arabian in its origin. Remarkably, some samples of this breed were grouped in the same genetic cluster as the Barb horse.

Discussion

The observed genetic diversity of the Marismeño horse indicates that the heterozygosity levels have remained similar to those observed for other Spanish horses or worldwide breeds (Vega-Pla et al. 2006), including Chinese (Ling et al. 2010) or American breeds (Conant et al. 2012), and it is also similar to the levels observed for donkeys (Jordana et al. 2016), Mediterranean horses (Marletta et al. 2006), Hispano-Breton (Pérez-Gutiérrez et al. 2008) and Italian horses (Zuccaro et al. 2008).

The allelic richness value exceeded the values obtained for 24 European native horse breeds (Warmuth et al. 2011) and for American donkeys (Jordana et al. 2016), in spite of being very similar to that found for 27 Chinese equine breeds (Ling et al. 2010), six Mediterranean horse breeds (Marletta et al. 2006) and some Italian horses (Zuccaro et al. 2008; Criscione et al. 2015). The observed differentiation levels among the horse breeds of this study can be considered low when compared with other domestic species (Martınez et al. 2000; Martínez et al. 2006; Delgado et al. 2012), but were similar to those obtained in relation to other horse studies (Cañon et al. 2000; Aberle et al. 2004; Glowatzki-Mullis et al. 2006; Felicetti et al. 2010; Warmuth et al. 2011). However, these levels were higher than those found by Aranguren-Méndez et al. (2001) and Jordana et al. (2016) with respect to donkeys.

The relationship analysis provided the expected results. Spanish Pure Breed stallions have been used to breed horses from the Guadalquivir marshes until a few years ago, before the establishment of the Studbook of the Marismeño breed in 2012. As a
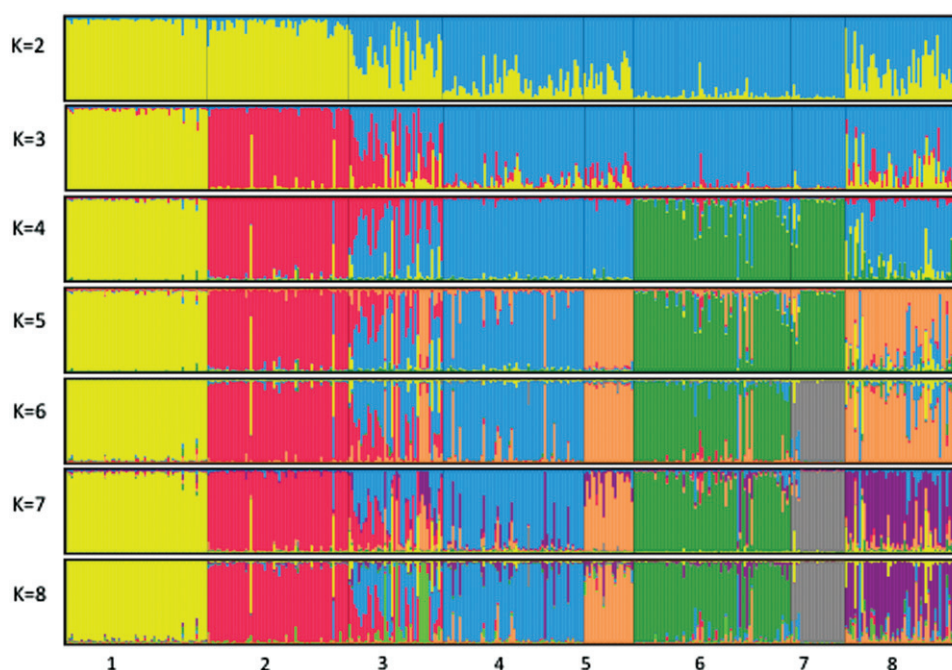

Figure 2. Neighbour-joining tree built from Nei's Da distance among the eight horse breeds.

Figure 3. Graphs of the individual Q-matrices obtained with the software STRUCTURE for K = 2 up to K = 9. Individuals ordered by breeds. Breed codes: 1.- Thoroughbred; 2.- Arabian; 3.- Hispano-Arabian; 4.- Spanish Purebred; 5.- Barb; 6.- Retuertas; 7.- Sorraia; 8.- Marismeño

consequence, a close genetic relationship between these breeds would be expected. The resulting neighbour-joining tree and the Structure clustering both supported this crossbreeding practise.

It was also noteworthy to observe some relatedness between the Marismeño and the Barb horses, which was probably due to the introduction in Andalusia of Barb horses during the Arabian invasion (which lasted for almost a millennium) (Muñoz-Bort 2004). It has not been well established where the Barb horse developed; some authors believe the breed could have originated in Northern Africa (Aparicio 1944). It seems that in prehistoric times and before the Greek and the Phoenician colonies, horses existing south of the Iberian Peninsula and the North African horses were very related, and a significant horse exchange existed between North Africa and the Iberian Peninsula, at least since Roman times and up to the Muslim period (Aparicio 1944). Subsequently, Iberian horses were crossbred with other European breeds to improve them and became what is currently the modern Spanish Purebred horse (Nissen 1963). Nevertheless, the Marismeño breed could have kept some genetic traces of the ancient crosses with the North African horses.

Interestingly, the expected outcome of the Marismeño phenogram was that the Marismeño horse would be closely related to the Retuertas breed because they are known to share a common ancestral origin. However, the Retuertas horse suffered from a strong bottleneck 30 years ago, which could in principle explain the high genetic distance found with the Marismeño horse. Moreover, the separation of the breeds seems to be the result of local horse breeders having systematically avoided for years crossing their mares with the local stallions, showing a morphology that fits with the ancient horse model resembling that of the Retuertas horses. A further line of evidence for the above arguments is the individual clustering obtained using a Bayesian approach, which indicates that the results of horse breeds that are not outbreed (i.e. fits well in its own cluster) was similar to a report for the Arabians and Thoroughbreds (Glowatzki-Mullis et al. 2006).

Currently, a crossbreeding between the Marismeño and the Retuertas horses would not be easy given that the Retuertas horse is confined to the Doñana Biological Reserve, an isolated area of 10,000 ha located within the Doñana National Park. Nevertheless, the possibility of incorporating horses from the Retuertas population in a future Marismeño conservation programme after an extensive morphological and genetic characterisation could be considered. Oddly, the Retuertas horse is in contrast with the Sorraia, probably due to a common geographic origin before the current reproductive isolation, although the Sorraia is markedly separated from the remaining breeds due to its reduced variability, which has already been observed in previous studies (e.g. Morais et al. 2005).

In conclusion, the Marismeño horse derives from the ancient horses, inhabiting the Guadalquivir marshes such as the Retuertas horses. In spite of the fact that some other breeds may have been introduced in more recent years, the results of this study indicate the uniqueness of this population adapted to this extreme environment. The management of the Marismeño horse studbook was officially established in 2012 avoiding new horse breeds influences, also the individual assignment of the foals to the population based on the genetic formula is considered in the conservation programme. Whether this is sufficient to ensure its future survival remains to be seen, but it clearly indicates that because these horses live with a minima human intervention, protecting the natural habitat of this population plays a major impact in its survival.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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