Saving feral horse populations: does it really matter? A case study of wild horses from Doñana National Park in southern Spain

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Summary

In the 1980s, a conservation programme involving a feral horse population, the Retuertas horses from the Guadalquivir marshes, was started in the Doñana National Park. The analysis of an extensive genetic survey of this population, which now numbers 100 animals, and 10 additional European and North African breeds using DNA polymorphisms from 22 microsatellites is presented. Highly significant fixation indexes were obtained for all pairwise comparisons between the Retuertas population and other breeds. A population neighbour-joining breed phenogram was built using different distance measures, but the Retuertas population failed to cluster with either of the two major clades of European and North African breeds, highlighting its uniqueness. In fact, the Retuertas population was positioned at the base of the trees, which were rooted using donkey samples. Furthermore, assignment tests and the individual Q-matrices obtained with the STRUCTURE programme isolated the Retuertas breed from the other breeds with only four K groups. Interestingly, some local semi-feral horses, known as Marismeño, also currently living in the Guadalquivir marshes, have some microsatellite genotypes that fall well within the Retuertas cluster. This raises the possibility of incorporating horses from the Marismeño population in a future conservation programme.

Keywords biochemical polymorphisms, correspondence analysis, genetic distance, horse, microsatellite, phylogeny.

Introduction

It is not known how long the Doñana feral horses known as Retuertas have lived in an area covering approximately 300 km^2 in the Doñana National Park (PND) in southern Spain, but oral and written accounts speculate that they have been in the Guadalquivir marshes since recorded history (Muñoz-Bort 2004). However, it is widely accepted that ancient wild horse populations almost disappeared from the North Iberian Peninsula and Europe during the Mesolithic period and were not reintroduced to the North of Iberia until the 8th century BC as a domestic animal (Cañon et al. 2000). There are no written records about the origin of the Retuertas, and little is known from a scientific point of view about wild horse populations in southern Spain.

In the 1980s, a programme of conservation of this feral horse population in the Doñana National Park was started. They grazed in the Guadalquivir marshes among a semi-feral horse population, known as Marismeños, which has traditionally been managed by local farmers who have systematically avoided crosses of feral Retuertas stallions with Marismeño mares. It is not known if the horses that were rescued for conservation by scientists on the Doñana Biological Reserve (RBD) look like those that grazed in southern Spain years ago. The morphological and functional features of Retuertas (e.g. an average height of only 1.42 m and adaptability to hostile marsh environment) are very different from Spanish Pure Breed, Arabian and other Iberian horses of Celtic origin, suggesting genetic isolation of the Retuertas horse population. These circumstances prompted us to compare the Retuertas horse population with the breeds cited above, including Marismeños.

Microsatellite markers are nowadays widely used to quantify genetic variation within and among horse breeds

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and to assign individuals to reference populations (Cañon et al. 2000; Glowatzki-Mullis et al. 2005). Therefore, we compared 22 microsatellite loci in the Retuertas horses and other Iberian, European and North African populations.

Materials and methods

Sample collection

Fresh blood samples of 55 Retuertas horses (RET) were obtained from the nucleus of the herd in the Doñana Biological Reserve. Blood and hair samples from an additional 449 horses were collected from 11 breeds, including Spanish Pure Breed (SPB, 60; also known as Andalusian Horse), Thoroughbred (THB, 46), Arabian (ARB, 48), Losino (LOS, 58), Asturcón (AST, 39), Mallorquín (MAL, 30), Menorquín (MEN, 69), Potok (POT, 27), Spanish Trotter (TRO, 46) and Marismeño (MAR, 26). Donkey samples (Equus asinus) (DNK, 44) were collected as an outgroup.

Serum protein and microsatellite typing

Five serum proteins (A1B-glycoprotein, albumin, carboxilesterase, vitamin D-binding protein and transferrin) were typed on the horse samples using one-dimensional PAGE according to Gahne et al. (1977). Following whole DNA preparation, samples were PCR amplified for 22 microsatellite loci: AHT4, AHT5 (Binns et al. 1995), ASB2, ASB17 (Breen et al. 1997), ASB23 (Lear et al. 1999), HMS3, HMS6, HMS7 (Guérin et al. 1994), HTG4 (Ellegren et al. 1992), HTG10 (Marklund et al. 1994), LEX33 (Shiue et al. 1999), TKY279, TKY287, TKY294, TKY297, TKY301, TKY312, TKY321, TKY325, TKY333, TKY337 and TKY341 (Tozaky et al. 2001). Sizing of PCR products was accomplished using an internal size standard and a reference sample on each gel.

Data analysis

FSTAT v2.9.3.2 (Goudet 1995) was used to calculate fixation indexes, allele numbers, and observed and expected heterozygosities. Levels of breed differentiation were estimated by calculating F_{ST} across breeds. Within-breed diversity (donkey excluded) was investigated by calculating allele number, observed and unbiased expected estimates of gene diversity within breeds (Nei 1973) and their standard deviations using Excel Microsatellite Toolkit software (Park 2001). Distribution of gene variability within and between breeds was studied by analysing Wright's (1951) F-statistics (Weir & Cockerham 1984; Excoffier et al. 1992) as implemented in GENETIX v4.04 (http://www.univ-montp2.fr/~genetix/genetix/genetix.htm). The inbreeding coefficient (F_{IS}) across populations was calculated with a 95% confidence interval determined using 1000 permutations and 10 000 bootstraps across loci.

Samples were tested for linkage disequilibria and departures from Hardy–Weinberg equilibrium by the Markov chain method. Heterogeneity in genotype distribution for all loci and all pairwise comparisons was tested based on an assumption of no differentiation, and it was implemented in GENEPOP v3.4 (Raymond & Rousset 1995).

Because drift and mutation influence differences between populations with long-term reproductive isolation, and because populations of recent divergence are probably mostly influenced by drift (Slatkin 1995), we followed two approaches in investigating the amplitude of genetic differentiation between breeds. Firstly, the computer program ARLEQUIN 3.01 (http://lgb.unige.ch/arlequin/) was used to calculate pairwise fixation indices based on allele frequency variation using an AMOVA framework to estimate weighted F_{ST} statistics (h) over all loci. Secondly, we calculated q (an R_{ST} analog), which is based on the stepwise mutation model (SMM) and takes into account differences in sample size and differences in variance between loci using R_{ST}CALC (Goodman 1997). The significance of genetic subdivision was assessed using 1000 permutations in both ARLEQUIN and R_{ST}CALC. To correct for multiple simultaneous comparisons, sequential Bonferroni corrections were applied to all pairwise tests using a global significance level of 0.05 (Rice 1989).

Genetic divergence among breeds was estimated by two approaches because of debate on model-specific distance estimators for microsatellite loci (e.g. Takezaki & Nei 1996; Goodman 1997; Angers & Bernatchez 1998). We first quantified Cavalli-Sforza & Edwards' (1967) chord distance (D_{CE}) and Nei's distance D_A (Nei 1983) using GENEDIST. We also calculated Goldstein et al.'s (1995) delta-mu squared $(d\mu)^2$ pairwise distances using R_{ST}CALC (Goodman 1997). Phenograms based on Cavalli-Sforza and Edwards' distance (D_{CE}), Nei's distance (D_A) and delta-mu squared $(d\mu)^2$ were constructed using the NEIGHBOR programme with the neighbor-joining algorithm (Saitou & Nei 1987). An additional phenogram was estimated using a maximum likelihood algorithm (CONTML). Support for the tree nodes was assessed by 10 000 bootstraps of gene frequencies using the SEQBOOT programme and compiled using the CONSENSE programme. All these programmes are included in the PHYLIP computer package, version 3.57c (Felsenstein 1995). Furthermore, the STRUCTURE v.2.1 computer program (Pritchard et al. 2000) was used to calculate clustering at different K values, which is the number of assumed populations using the admixture model in which an individual may have mixed ancestry. It is based on a Bayesian clustering algorithm that uses multilocus genotypes to infer population structure and to assign individuals to populations. We performed runs of 10^6 iterations, following a burn-in period of 100 000 iterations. To determine the adequate number of inferred clusters to fit the data, we evaluated K-values from 2 to 14 and ran three independent simulations of this length for each K to evaluate stability. Graphics from

these results were drawn using DISTRUCT software (http://rosenberglab.bioinformatics.med.umich.edu/distruct.html).

Factorial correspondence analysis (Lebart et al. 1984) was performed to test for possible admixture between the Retuertas and other populations using the module 'AFD sub populations' of the GENETIX v4.04 software. This method does not depend on the mutation model used for markers (Laloe et al. 2002), which is advantageous for analysis of microsatellites because knowledge of their mutation process is insufficient (Estoup & Cornuet 1999). Concordance among different methods to study the genetic differentiation between individuals or populations highlights the robustness of the results (MacHugh et al. 1997).

Results

Protein polymorphisms

Of the five protein systems analysed, A1B-glycoprotein, albumin, vitamin D-binding and transferrin showed usual electrophoretic variants. However, the Retuertas population showed a carboxilesterase allele that has not previously been recorded for any horse blood typing lab in any horse comparison test organized by the International Society for Animal Genetics (ISAG). The new allele, provisionally named m, occurs in this population at a frequency of 0.16.

Microsatellite diversity and test of disequilibria

All microsatellite markers showed high levels of polymorphism, with an average of 10.95 alleles per locus, and all breeds compared were genetically diverse, showing high heterozygosities at most loci (Table S1). A mean of 7.07 alleles per population were detected; the Retuertas and Thoroughbred populations showed the lowest number of alleles (5.91 and 5.93 respectively). Observed and expected heterozygosities in each breed and their respective standard errors are shown in Table 1. Gene diversity indicated that, despite selective breeding programmes for most of these breeds, levels of heterozygosity remain similar to those reported in other populations of large wild herbivorous mammals (e.g. Polziehn et al. 2000; Bonnet et al. 2002). The sample of Arabian horses (ARA) showed a significant deficiency of heterozygosity. In contrast, the Retuertas (RET) population showed a significant excess of heterozygosity and the lowest F_{IS} value. Negative F_{IS} values were also found in other endangered breeds, but these values were not significant. Furthermore, only 13 significant departures from Hardy–Weinberg equilibrium were detected among 242 population-locus combinations, precisely the number expected by chance at the 5% level. No population showed more than three markers deviating from Hardy–Weinberg equilibrium.

Breed differentiation and genetic distances

The average F-statistics and their 95% confidence intervals obtained with 10 000 bootstraps over loci were F_{IS} ¼)0.008 ()0.0208–0.0048), F_{IT} ¼ 0.0848 (0.072–0.097) and F_{ST} ¼ 0.091 (0.084–0.099). However, all comparisons between pairs of breeds gave fixation indices significantly different from zero, irrespective of whether the index was based on the infinite alleles model (h) or the stepwise mutation model (q) (Table 2). Although not fully resolved, dendrograms depicted four clusters of closely related breeds with bootstrap support above 50%, irrespective of whether they were based on the maximum likelihood or Neighbour-Joining algorithms using chord distance (D_{CE}) or Nei's

Table 1 Sample sizes, number of alleles, heterozygosities and inbreeding coefficients.

Breed[1]	Sample size	No. alleles (SD)	Unbiased heterozygosity (SD)	Observed heterozygosity (SD)	Inbreeding coefficient (F_{IS})
RET	55	5.91 (1.06)	0.6857 (0.0194)	0.7255 (0.0129)	)0.0587*
ARA	48	6.05 (1.50)	0.6725 (0.0245)	0.6353 (0.0148)	0.0559*
AST	39	7.18 (1.74)	0.7361 (0.0195)	0.7584 (0.0146)	)0.0307
SPB	60	7.77 (1.34)	0.7522 (0.0171)	0.7477 (0.0120)	0.0060
THB	46	5.73 (1.08)	0.7350 (0.0128)	0.7418 (0.0138)	)0.0093
LOS	59	8.27 (1.52)	0.7444 (0.0169)	0.7522 (0.0120)	)0.0105
MAL	30	6.50 (1.26)	0.7354 (0.0212)	0.7583 (0.0167)	)0.0318
MAR	26	7.68 (1.25)	0.7951 (0.0087)	0.7863 (0.0172)	0.0113
MEN	69	7.50 (1.65)	0.7450 (0.0163)	0.7483 (0.0114)	)0.0014
POT	29	7.86 (2.12)	0.7777 (0.0261)	0.7815 (0.0168)	)0.0049
TRO	46	7.27 (1.08)	0.7593 (0.0145)	0.7520 (0.0136)	0.0097

SD, standard deviation; *Significant values for heterozygous deficit or excess ($P < 0.05$)
[1]Breed abbreviations: RET, Retuertas; ARA, Arabian; AST, Asturcón; SPB, Spanish Pure Breed (also known as Andalusian Horse); THB, Thoroughbred; LOS, Losino; MAL, Mallorquín; MAR, Marismeño; MEN, Menorquín; POT, Potok; TRO, Spanish Trotter.

Table 2 Pairwise fixation indices for all horse breed pairs and donkeys.

Breed[1]	RET	ARA	AST	SPB	THB	LOS	MAL	MAR	MEN	POT	TRO	DNK
RET		0.149	0.113	0.091	0.160	0.108	0.122	0.068	0.117	0.110	0.190	0.345
ARA	0.161		0.082	0.101	0.124	0.086	0.102	0.066	0.051	0.063	0.087	0.788
AST	0.166	0.109		0.109	0.189	0.039	0.101	0.080	0.039	0.052	0.118	0.334
SPB	0.106	0.108	0.079		0.103	0.066	0.078	0.021	0.030	0.090	0.134	0.314
THB	0.181	0.134	0.122	0.095		0.137	0.116	0.076	0.127	0.127	0.066	0.337
LOS	0.160	0.113	0.059	0.067	0.118		0.071	0.080	0.001	0.048	0.082	0.318
MAL	0.156	0.127	0.087	0.076	0.117	0.088		0.055	0.074	0.087	0.110	0.352
MAR	0.070	0.078	0.047	0.015	0.068	0.044	0.056		0.059	0.045	0.098	0.313
MEN	0.146	0.118	0.079	0.063	0.119	0.070	0.066	0.050		0.059	0.077	0.320
POT	0.160	0.118	0.062	0.062	0.092	0.040	0.072	0.039	0.071		0.074	0.332
TRO	0.127	0.113	0.087	0.074	0.072	0.084	0.080	0.051	0.082	0.058		0.328
DNK	0.790	0.379	0.769	0.782	0.775	0.775	0.787	0.743	0.770	0.742	0.756	

Fixation indices were computed following the infinite allele model (h; lower diagonal), and the unbiased estimator of Slatkin's R_{ST} (q; Goodman 1997; upper diagonal).

[1]Breed abbreviations: RET, Retuertas; ARA, Arabian; AST, Asturcón; SPB, Spanish Pure Breed (also known as Andalusian Horse); THB, Thoroughbred; LOS, Losino; MAL, Mallorquín; MAR, Marismeño; MEN, Menorquín; POT, Potok; TRO, Spanish Trotter; DNK, Donkey.

Figure 1 Neighbour-joining tree built from D_A distances of eleven horse populations including a sample of donkey to root the tree. Numbers indicate percentage of 1000 bootstrap replications across loci.

distance (Da). Thus, only Nei's (Da) distance is presented (Fig. 1). Interestingly, the Retuertas population did not cluster with any of these European breeds and fell in a dichotomy with the outgroup, with bootstrap support above 70%. One cluster grouped Atlantic breeds of Celtic origin (Asturcón, Potok and Losino) and another of Mediterranean breeds of the same origin (Menorquín and Mallorquín). Bootstrap support varied from 42% to 78% for the internal nodes within this first main cluster. The smallest distance was between the Marismeño population and Spanish Pure Breed. This is not surprising as the Marismeño has traditionally been crossbred with Spanish Pure Breed stallions, and the Marismeño population is closely related to the Spanish Pure Breed horse. Bootstrap support for this cluster was 95%. Spanish Trotter showed an expected relationship with Thoroughbred. The Retuertas population did not cluster with either of these four major clades, and its location is alone near the root with relatively high bootstrap support, which further substantiates the uniqueness of these horses.

The STRUCTURE programmes showed that the independent runs from K ¼ 2 to K ¼ 14 produced consistent results. Clustering was performed on the entire data set with increasing numbers of inferred clusters. The assignment test results determined that 12 breeds best fit the data and that the Losino and Pottok horses appear admixed. A plot of the clustering results for the individuals in the sample is shown in Fig. 2. K ¼ 2 indicated the presence of two very distinct clusters, corresponding to the donkey and horses. From sample K ¼ 4–12, assignments reflected the presence of population structure associated with progressive genetic differentiation (e.g. autochthonous Spanish breeds versus international breeds, Celtic ponies versus Mediterranean breeds, etc.). Assignment tests isolated the Retuertas horse from the other breeds as early as four K groups, and this group maintained its integrity throughout the analysis. It was clearly differentiated from its neighbour, the Marismeño, and all other Spanish breeds.

Factorial correspondence analysis

Correspondence analysis was further used to detect admixtures between the Retuertas and the rest of the populations (Fig. 3). The first axis accounted for 19.93% of the total inertia proportion, while the second axis accounted for 17.05%. This analysis also placed the Retuertas horses in a cluster, thereby further substantiating its singularity.

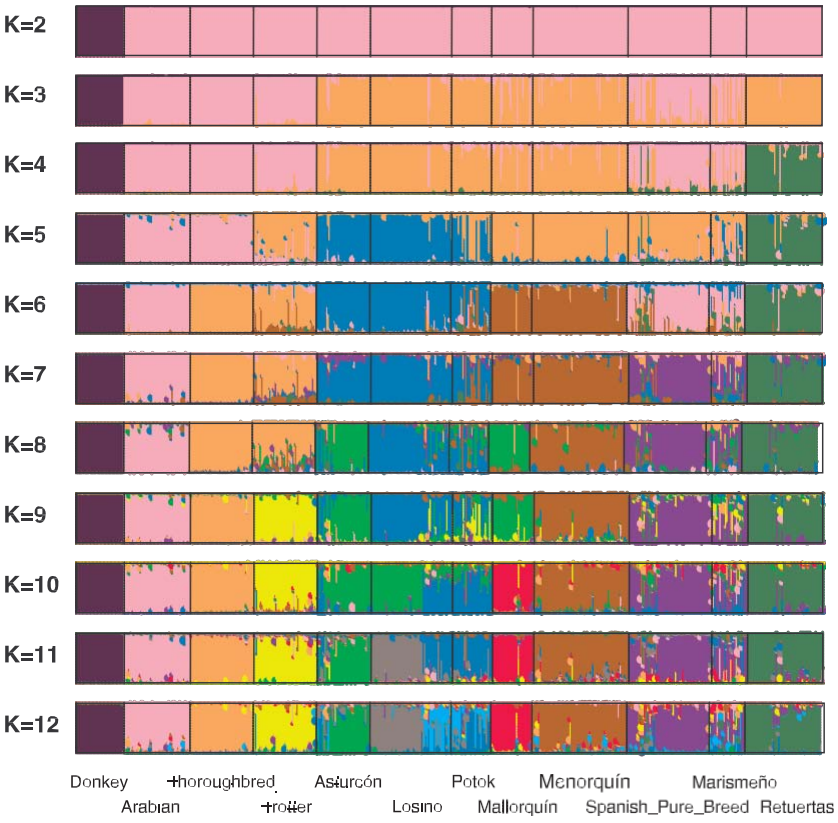

Figure 2 Graphs of individual Q-matrices obtained with STRUCTURE programme for K ¼ 2–12. Individuals were ordered by populations and separators between them were included.

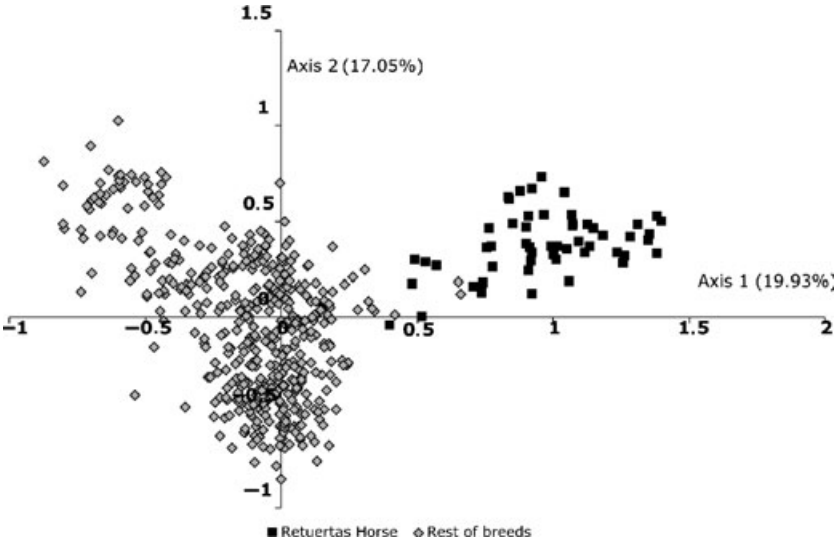

Figure 3 Factorial correspondence scatter plot of the first and second axes.

Discussion

The finding of a previously unknown allele of a major protein such as carboxilesterase indicates the uniqueness and genetic isolation of the Retuertas horse population. The presence of this new allele could be the result of either a rare ancestral polymorphism, which, due to long-term reproductive isolation of these horses, is absent in all other modern breeds so far characterised, or represents a new mutation in this isolated gene pool. The latter possibility is remote because the average nucleotide substitution rate for non-synonymous sites is 0.74 ± 0.67 per site per 10^9 years. Even in the most variable genes so far characterized, such as MHC genes, the nucleotide substitution rate at non-synonymous sites was only 3.06 ± 0.37 per site per 10^9 years (Graur & Li 1999). However, confirmation of these hypotheses requires further testing.

The observed levels of differentiation among the horse breeds are low when compared with other domestic species (e.g. Hanslik et al. 2000; Martínez et al. 2000), but similar to those obtained in other horse studies (Bjørnstad et al. 2000; Cañon et al. 2000; Bjørnstad & Røed 2002; Aberle et al. 2004; Glowatzki-Mullis et al. 2005) and higher than those obtained by Aranguren-Méndez et al. (2001) in donkeys. Furthermore, levels of genetic diversity are also similar to those obtained in the above horse breed studies. Fixation indices based on the infinite allele model (h) or the stepwise mutation model (q) were of the same order of magnitude. These results indicated that both drift and mutation probably influence the observed levels of divergence. To illustrate how time of divergence affects these estimators, one can refer to the h and q pairwise values between horses and donkeys. For h values, they are around 30%, while q values are above 70%, reflecting the thousands of generations of divergence between horses and donkeys. In this case, it is expected that the q estimator better reflects the magnitude of divergence between species in the absence of gene flow (Slatkin 1995; Balloux & Goudet 2002).

The relationship analysis provided a rather surprising result. The logically expected outcome of the breed phenogram was that the Retuertas horse would fall close to the Marismeño and Spanish Pure Breed, as these populations share a common geographic area and habitat in semi-feral conditions. Therefore, the eventual crossbreeding of these populations would have been possible. However, local horse breeders have systematically avoided crossing their mares and stallions with the feral populations of Retuertas horses, and whenever an accidental crossing has taken place, the foals were taken out of the Marismeño population. This result is well supported by all methods of tree building and, even when poor breed differentiation causing low bootstrap values was observed in many nodes, the Retuertas population showed 73% of robustness of the node where it was situated.

A further line of evidence for the above arguments is the individual clustering obtained using a Bayesian approach, which indicates that horse breeds that are not outbreed fit well in their own cluster, a result similar to a recent report for Arabians and Thoroughbreds (Glowatzki-Mullis et al. 2005). The Retuertas horses also clustered in a unified population from early runs of the analysis, substantiating its integrity and uniqueness. Interestingly, some horses currently living in the Guadalquivir marshes among Marismeño horses have a microsatellite multilocus genotype that falls well within the Retuertas cluster. This raises the possibility of incorporating horses from the Marismeño population in a future conservation programme after extensive morphological and genetic characterization, which will result in an increased effective population size.

These results raise the possibility that the Retuertas horse population has remained reproductively isolated from most domestic breeds for a long time and may well be a direct descendent of wild horses that have survived in the Guadalquivir marshes for centuries. Sequencing and phylogenetic analyses of genes and the control region from mtDNA have shown evidence of multiple events of domestication in the Old World in many distinct geographic areas followed by extensive gene flow leading to incomplete lineage sorting and extensive sharing of haplotypes among breeds (Vila et al. 2001; Hill et al. 2002; Jansen et al. 2002; Bruford et al. 2003). Therefore, analysis of mtDNA of the Retuertas population would result in inconclusive evidence as well.

A last issue raised by these results refers to the long-standing debate on minimum viable population size (e.g. Franklin 1980; Connor & Simberloff 1986; Lande & Barrowclough 1987; Walters 1991; Craig 1994). The widely accepted view at present suggests that there is no single number that tips a species into extinction or survival but that a number of demographic, stochastic and genetic variance factors play fundamental roles in the fate of small populations. The present Retuertas horse population shows no signs of inbreeding depression and has recovered from only some tens of horses. Whether this is sufficient to ensure its future survival remains to be seen, but it clearly indicates that because these horses were introduced into a national park and left to live with no human intervention, protecting the natural habitat of a population plays a major impact in its survival.

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Supplementary Material

The following supplementary material is available for this article online from http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2052.2006.01533.x

Table S1 Number of alleles, unbiased heterozygosity, observed heterocygosity, genetic differentiation index (F_{ST}) and inbreeding coefficient (F_{IS}).

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