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Research Article

Genetic characterization of the Spanish Trotter horse breed using microsatellite markers

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Abstract

To assist in selection schemes we carried out the first genetic characterization of the Spanish Trotter horse (Trotador Español). We used 16 microsatellite markers to genotype 40 unrelated Spanish trotters, 25 native Balearic horses (11 Menorquina and 14 Mallorquina horses) and 32 Andalusian horses. The observed heterozygosity for the Spanish Trotters was 0.647 ± 0.037 and the expected heterozygosity was 0.696 ± 0.026 while the average number of alleles per locus was 6.0 ± 0.341, these values being similar to the data published for other horse breeds. We also tried to establish the importance of the Mallorquina and Menorquina breeds in the present Spanish Trotter population. Only 9% of the total genetic variability could be attributed to differences between breeds (mean F_{ST} = 0.09 ± 0.010). Recent migration rates were confirmed the low recent genetic relationship between the Balearic breeds and Spanish Trotters, indicating that the genetic background of the present Spanish Trotter population is not based on the native Balearic horse population.

Key words: horse, Spanish Trotter breed, microsatellites, genetic diferenciation, migration rates.

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Introduction

The Spanish Trotter horse (*Equus caballus*), also known in spanish language as *Trotador Español*, is a breed of increasing importance in Spain. The Gran Premio Nacional (Spanish national harness-race) is a Trotter horse race which is a prestigious event in the Spanish horse industry and is evidence of the importance of this breed.

The Spanish Trotter studbook was founded in 1980 and at present includes 15,510 horses (Azor *et al*., 2005; unpublished data). The Spanish Trotter population is mainly bred in the Balearic Islands of Majorca (Mallorca in Spanish), Minorca (Menorca in Spanish) and Ibiza, the latest census showing that 85% of the Spanish Trotter population is located in Majorca. Artificial insemination is the most frequent reproductive practice used in the breed, with semen mainly selected from French and American Trotter stallions. The foals with French or American Trotter stallions are registered in the Spanish Trotter studbook including the identification and the origin of the parent.

Historically, the Spanish Trotter breed was formed by mating native Balearic mares belonging to the Mallorquina breed from Majorca island and the Menorquina breed from Minorca with foreign stallions, mainly from Russian Orlov Trotters and French Trotters (Llamas *et al*., 1992). The Mallorquina and Menorquina breeds are two endangered horse populations included in the Food and Agricultural Organization of the United Nations (FAO) list of domestic animals to be protected FAO, http://dad.fao.org/es/home.htm.

The Mallorquina and Menorquina studbooks were founded in 1993 and the genetic admixture between them is considered important (Cañón *et al.* 2000). The Mallorquina studbook contains 247 horses while the Menorquina studbook contains 2,351 horses, although a census conducted in 2003 by the Spanish Ministry of Agriculture, Fisheries and Food "Estudio y caracterización del sector equino en España" (http://www.mapa.es/app/Equino/documentos/Estudio%20y%20Caracterización%20del%20Sector%20Equino%20en%20España.pdf) recorded only 172 Mallorquina and 1,647 Menorquina horses.

The Spanish Trotter Breeders Association (ASTROT) has recently implemented a selection scheme with the support of Spanish Ministry of Agriculture, Fisheries and Food and has tried to establish the importance of the native Balearic Mallorquina and Menorquina breeds in the present Spanish Trotter population. However, there

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have been only a few molecular studies of Iberian horses (Vila *et al*., 2001; Lopes *et al*., 2005; Royo *et al*., 2005[a]) especially using microsatellites (Cañón *et al*., 2000) and in this context the molecular characterization of the Spanish Trotter breed would is important.

Material and Methods

Sample collection and DNA isolation

Tubes containing ethylenediaminetetraacetic acid (EDTA) were used to collect blood samples from 40 unrelated horses registered in the Spanish Trotter studbook. Genealogical information showed that the ancestry of 11 of the Spanish Trotters was more than 75% American Trotter while that of a further 15 of the Spanish Trotters was more than 75% French Trotter. We also collected blood samples from 25 unrelated native Balearic horses (14 Menorquina and 11 Mallorquina). Blood samples were also collected from an outgroup of 32 Andalusian horses. Genomic DNA was extracted from whole blood using the salting out procedure of Miller *et al*. 1988.

Microsatellite amplification

Samples were genotyped for a set of 16 microsatellites (Table 1) recommended for paternity tests and individual identification by the International Society for Animal Genetics (ISAG). Microsatellites were amplified using fluorescently-labeled primers (StockMarks® for horses, PE Applied Biosystems, Foster City, CA) following the PCR conditions given by Dimsoski (2003) and the PCR was run on a Mastercycler® ep gradient S thermal cycler (Eppendorf, Germany) using 10 min at 95 °C to activate the AmpliTaq Gold DNA polymerase followed by 30 cycles of 30 s at 95, °ºC, 30 s at 60 °C and 60 s at 72 °C, with a final extension of 60 min at 72 °C. The PCR products were stored frozen until they were detected by capillary electrophoresis using an Applied Biosystems 3100 DNA sequencer. Allele sizes were determined after processing the raw data with the software packages *GeneScan 3.7* and *Genotyper 3.7* using a LIZ 500 bp internal size standard (Applied Biosystems).

Statistical analysis

The *Genetix 4.2* program (Belkhir *et al*. 2001) was used to compute the following parameters across loci and populations: allele frequencies, number of alleles per locus, observed heterozygosity (Ho) and expected heterozygosity (He). This program was also used to compute Wright's F statistics (F_{ST}, F_{IS} and F_{IT}; Wright, 1965, 1978) in the form proposed by Weir and Cockerham (1984). The different

Table 1 - Total number alleles (k), observed (Ho) and expected heterozygosity (He) and chromosome location of the microsatellites analyzed in the Spanish Trotter breed and in all the breeds analyzed.

	Total number alleles (k)		Heterozygosity				Chromosome location
	Spanish Trotter	Whole population	Spanish Trotter		Whole population		
Loci	k	k	Ho	He	Ho	He	
AHT4	5	8	0.675	0.686	0.694	0.754	24
AHT5	7	7	0.750	0.756	0.752	0.801	A8
ASB17	7	12	0.775	0.791	0.717	0.839	2
ASB2	8	8	0.793	0.765	0.805	0.802	15
ASB23	6	6	0.658	0.757	0.611	0.789	3
CA425	6	8	0.514	0.697	0.510	0.698	28
HMS1	6	6	0.553	0.708	0.522	0.649	15
HMS2	7	8	0.839	0.802	0.837	0.809	10
HMS3	5	7	0.538	0.543	0.541	0.686	9
HMS6	5	6	0.842	0.731	0.743	0.763	4
HMS7	5	8	0.500	0.646	0.666	0.777	1
HTG10	8	11	0.571	0.707	0.729	0.871	21
HTG4	5	6	0.700	0.720	0.733	0.759	9
HTG6	4	6	0.650	0.628	0.571	0.676	15
HTG7	4	5	0.275	0.399	0.347	0.464	4
VHL20	8	9	0.725	0.793	0.670	0.837	30
All loci	96	121	0.647	0.696	0.653	0.748	

The 16 loci used were: AHT4, AHT5 (Binns *et al.* 1995); ASB17, ASB2, (Breen *et al*., 1997); ASB23 (Lear *et al*. 1999); UCDEQ425 (Eggleston-Stott *et al*., 1997); HMS1, HMS2, HMS6, HMS7 (Guerín *et al*, 1994); HTG4, HTG6 (Ellegren *et al.* 1992), HMS3, HTG7, HTG10 (Marklund *et al.*, 1994); and VHL20 (Van Haeringen *et al*., 1994).

F-statistics look at different levels of population structure. F_{IT} is the inbreeding coefficient of an individual (I) relative to the total (T) population, F_{IS} is the inbreeding coefficient of an individual (I) relative to the subpopulation (S) and F_{ST} is the effect of subpopulations (S) compared to the total population (T), and is calculated by solving the equation $(1 - F_{IS})(1 - F_{ST}) = (1 - F_{IT})$.

We also used the *Genetix 4.2* program to compute the among population Reynolds' distance matrix (Reynolds *et al.* 1983) and the effective number of migrants per generation (*Nm*; Wright, 1969). The number of migrants per generation (*Nm*) can be interpreted as the upper limit of the number of migrants per generation allowing for the maintenance of the observed genetic differentiation between the breeds, the more divergent the populations the lower the *Nm* value, (Slatkin, 1985). Individual multilocus genotypes were also investigated by carrying out a factorial analysis of correspondences to obtain an unbiased test of population structure.

Recent migration rates among populations were estimated using the *BayesAss+* program (Wilson and Rannala, 2003). This method puts less emphasis on some key assumptions from previous assignment methodologies, namely that genotypes are in Hardy-Weinberg equilibrium within populations. The program simultaneously estimates the probability distribution of allelic frequencies for each locus and migration rates between populations (*m*), assessing the relative importance of specific patterns of population dynamics. The *BayesAss+* program was ran using a burning and a data collection period of 3×10^6 iterations.

Results

Genetic diversity

Parameters characterizing the polymorphism of the microsatellite set used are given in Table 1. A total of 121 alleles were detected across the 16 loci analyzed. The number of alleles per marker varied from 5 for HTG7 to 12 for ASB17 and the across loci Ho value ranged from 0.347 for HTG7 to 0.837 for HMS2 while the He value varied from 0.464 for HTG7 to 0.871 for HTG10. The heterozygosity values for the whole dataset were 0.653 for Ho and 0.748 for He. The average number of alleles per locus ranged from 4.5 ± 0.532 for the Mallorquina breed to 6.0 ± 0.341 for the Spanish Trotters, while the highest heterozygosity values (Ho = 0.729; He = 0.725) were shown by the Menorquina horses and the lowest values (Ho = 0.618; He = 0.613) by the Andalusian horses (Table 2). The F_{IS} value varied from 0.051 for the Menorquina horses to 0.113 for the Mallorquina horses, demonstrating a clear heterozygote deficiency for all the breeds analyzed.

Population structure

The F-statistics values for the whole population (*i.e.* Spanish Trotters + native Balearic + Andalusian) were F_{IS} = 0.085 ± 0.022, F_{IT} = 0.167 ± 0.022 and F_{ST} = 0.090 ± 0.010. Differentiation between breeds was estimated using Reynolds' distance, the values of which varied from 0.001 for the Menorquina-Mallorquina pair to 0.126 for the Mallorquina-Andalusian pair (Table 3). Table 3 shows that the highest effective number of migrants per generation (*Nm* = 170.16) was between the Menorquina-Mallorquina pair, which was very high in comparison to the values for the Mallorquina-Spanish Trotter pair (*Nm* = 1.94) and the Mallorquina-Andalusian pair (*Nm* = 1.86).

Regarding the analysis of the individual horses, factorial analysis of correspondence identified three factors with an eigenvalue = 1 explaining 84.17% of the total variance. Figure 1 gives a plot showing the location of the analyzed horses in the bi-dimensional space formed by the two first factors (explaining, respectively 37.24% and 26.45% of the total variance). The X-axis dimension clearly differentiates

Table 3 - Reynolds distance Matrix (upper diagonal) and effective number of migrants per generation (*Nm,* lower diagonal) for the four breeds analyzed.

	Mallorquina	Menorquina	Spanish Trotter	Andalusian Horse
Mallorquina		0.001	0.121	0.126
Menorquina	170.16		0.062	0.080
Spanish Trotter	1.94	3.82		0.093
Andalusian Horse	1.86	2.98	2.55	

Table 2 - Number of samples (N), average number alleles per locus and breed (k), observed (Ho) and expected heterocygosity (He) and F_{IS}.parameter.

Population	N	k	Heterocygosity (± SE)[a]		F_{IS} ± SE
			Observed ± SE	Expected ± SE	
Mallorquina	11	4.5 ± 0.532	0.649 ± 0.071	0.604 ± 0.542	0.113 ± 0.059
Menorquina	14	5.7 ± 0.309	0.729 ± 0.042	0.725 ± 0.019	0.051 ± 0.045
Spanish Trotter	40	6.0 ± 0.341	0.647 ± 0.037	0.695 ± 0.026	0.091 ± 0.032
Andalusian Horse	32	5.1 ± 0.543	0.618 ± 0.060	0.613 ± 0.044	0.096 ± 0.038

[a]Standard error.

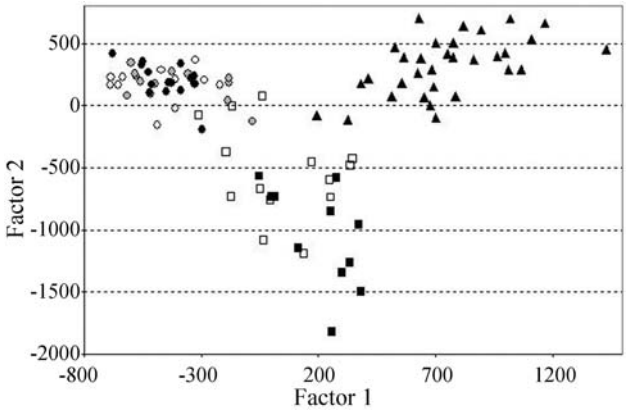

Figure 1 - Correspondence analysis using individual multilocus genotypes in the Spanish horse breeds analyzed. Individual Andalusian horses are represented by triangles, Mallorquina horses by black squares and Menorquina horses by open squares. Spanish Trotter horses of American Trotter ancestry are shown by gray circles, those of French Trotter ancestry by black circles and those with less than 75% of their ancestors from American and French Trotter horses by open circles.

the Andalusian horses from the rest while the Y-axis dimension separates the Spanish Trotter and Balearic horses, with the Balearics showing no clear differentiation between breeds. Estimates of recent migration rates between populations (up to the second generation of migrants) are shown in Table 4. Three of the breeds (Spanish Trotter, Menorquina, and Andalusian) have not received a significant proportion of migrants. Significant recent introgression rates (approximately 30%) were found for the Menorquina breed to Mallorquina breed. Average standard deviations of all distributions of *m* for each source population ranged from 0.004 to 0.048.

Discussion

During the research described in this paper we carried out the first genetic analysis of the Spanish Trotter breed using DNA markers. The polymorphism of the microsatellites reported in the literature for other horse breeds mostly range from 0.66 to 0.75 for Ho and from 0.64 to 0.77 for He (Wimmers *et al*., 1998; Bjørnstad *et al*., 2000; Cañón *et al*., 2000; Cunnnigham *et al*., 2001; Luís *et al*., 2002; Aberlé *et al*., 2004; Achmann *et al*., 2004; Galov *et al*., 2005; Royo *et al*., 2005[b]), although these values are not directly comparable with those detected by us because of differences in the microsatellite sets used.

Table 4 - Means of the posterior distribution of the estimated migration rates (*m*) for each population using the *BayesAss+* program. The rows show the origins of the migrants while the columns show the populations which individual horses are migrating to (derived populations). Values on the diagonal are the proportion of individuals derived from the source populations for each generation. Contributions higher than 0.10 are in bold.

	Derived population			
Parental population	Mallorquina	Menorquina	Spanish Trotter	Andalusian Horse
Mallorquina	**0.6963**	0.0218	0.0029	0.0105
Menorquina	**0.2680**	**0.8953**	0.0027	0.0302
Spanish Trotter	0.0179	0.0658	**0.9913**	0.0149
Andalusian Horse	0.0176	0.0169	0.0029	**0.9443**

The breeds analyzed by us showed strong heterozygote deficiency, characterized by high and positive F_{IS} values. Gómez *et al*. (2005) have pointed out that Spanish Trotters are under high selection pressure for racing performance while Valera *et al*. (2005) has reported that the Andalusian breed is under selection pressure for morphological traits. The frequent use of semen from selected foreign studs in the Spanish Trotter breed is also significant (Azor *et al*., 2005; unpublished data).

The overall F_{ST} value for our whole data set was similar to but slightly higher than the 7.8% reported by Cañón *et al*., (2000) for seven Spanish horse breeds (Asturcón, Caballo Galego, Losina, Pottoka, Jaca Navarra, Mallorquina and Menorquina) and the Thoroughbreds used as outgroup. However, our F_{ST} value was slightly smaller than that found in some other studies, such as the 10% reported by Zabek *et al*. (2005) for some Polish breeds (Bilgoraj, Malopolski and Thoroughbreds), the 10.9% reported by Díaz *et al*., (2002) for the Argentine Creole and Thoroughbreds and the 11.7% reported by Lippi and Mortari (2003) for two Brazilian breeds (Mangalarga Marchador and Mangalarga). It has been pointed out by some authors that the typical high within-population variability of microsatellites may result in low differentiation values (Hedrick, 1999; Balloux and Lugon-Moulin, 2002). Thus, the order of magnitude of genetic differentiation between breeds assessed by F_{ST} estimators seems to be always low and rather constant regardless of the species (MacHugh *et al*. 1998; Laval *et al*., 2000; Arranz *et al*., 2001).

We estimated short-term differentiation between breeds using Reynolds' distance, which has been shown to be an appropriate measure for livestock populations with short-term divergence (Reynolds *et al*., 1983; Laval *et al*., 2002; Álvarez *et al*., 2005).

As expected, the highest distance values in our study were for the Andalusian outgroup. The Spanish Trotters was more distant from the Mallorquina sample than from the Menorquina sample, which does not agree with the main geographical location of the populations in that 85% of the Spanish Trotter population is located in Majorca, the home of the Mallorquina breed.

In general, the *Nm* values for the Spanish Trotters paired with the other breeds were higher than for the Andalusian horses paired with the other breeds and this highlights the weak genetic relationship in recent times between Spanish Trotters and the native Balearic horses, contrasting with the high genetic relationships (*Nm* = 170.16) between the Mallorquina and Menorquina native Balearic breeds.

However, the *Nm* gene flow estimator is derived from simplified models of population structure that assume constant population sizes, symmetrical migration at constant rates and population persistence for periods sufficient to obtain genetic equilibrium (Slatkin, 1985) and that probably do not agree with the scenario analyzed in this study. There is a need to apply new and more informative methodologies to establish the evolutionary history of present populations both in the long term flow of genes and in the recent patterns of migration (Wilson and Rannala, 2003). The recent migration rates estimated using the *BayesAss+* program (0.0029 and 0.0027) support the low recent genetic relationship between Spanish Trotters and the native Balearic breeds sampled. These values of *m* were closed to zero and no significant. For individual horses this situation was also demonstrated by the correspondence analysis that enabled individual horses to be assigned without much uncertainty to their corresponding breeds, or, in the case of the native Balearic horses, breed group. However, correspondence analysis did not enable individual Spanish trotters to be distinguished according to their French or American origin.

Summarizing the information above, we can conclude that although originally the Spanish Trotter could have had a genetic background from Balearic native horses, it has been confirmed that recent generations have not been influenced by the native Balearic horses. However, further analysis will be needed to establish the level of genetic differentiation between the Spanish Trotter horse breed and other international Trotter breeds.

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