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GENETIC VARIABILITY OF SMALL HORSE POPULATIONS FROM GREEK ISLANDS

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Abstract: In this study, we analyzed microsatellite variation in DNA obtained from hair samples collected from 46 local Greek horse populations originating from the islands of Skyros (Skyros Small Horse; n=9), Rhodes (Rodos Small Horse; n=6), Lesvos (with the traditional miniature Midili Small Horse (n=2) and the larger Lesvos Gaiter (n=22)) and Crete (Messara) (n=7). We used 15 autosomal microsatellite markers (VHL20, HTG4, AHT4, HMS7, HTG6, AHT5, HMS6, ASB2, HTG10, HTG7, HMS3, HMS2, ASB17, ASB23 and LEX33) for the genetic characterization of the above populations and exploration of their genetic structure and diversity levels. A total of 120 alleles were detected across the 15 loci with a minimum of 4 alleles in HTG7 locus and a maximum of 13 alleles in ASB17 locus. The total per population number of alleles was 42 (Skyros Small Horse), 20 (Rodos Small Horse), 71 (Lesvos Gaiter), 52 (Messara breed) and 21 (Midili Small Horse). The effective number of alleles (N_e) per locus ranged from 1.47±0.13 (Rodos Small Horse) to 4.67±0.31 (Lesvos Gaiter). The allelic richness (A_r) was between 1.50±0.12 (Rodos Small Horse) and 2.93±0.08 (Lesvos Gaiter) and the average Polymorphism Information Content (PIC) values varied from 0.200±0.035 (Rodos Small Horse) to 0.733±0.026 (Lesvos Gaiter). No significant deviations from H-W equilibrium were found except for three loci (ASB2, HTG10 and LEX33) in Messara and one locus (ASB23) in Lesvos Gaiter. The inbreeding coefficient (F_{is}) ranged from -0.130 (Rodos Small Horse) to 0.042 (Lesvos Gaiter). The observed (H_o) and expected (H_e) multilocus heterozygosity mean estimations were highest in Lesvos Gaiter (0.764±0.027 and 0.783±0.024, respectively) and smallest in Rodos Small Horse (0.300±0.075 and 0.269±0.064, respectively). Across loci, the total genetic diversity H_T was 0.741, the diversity among subpopulations H_S was 0.621 and the multilocus genetic differentiation G_{ST} was 0.161, which was rather high. The population of Rodos Small Horse separated from the remaining horses as shown by factorial correspondence analysis, population assignment and metric multidimensional scaling diagrams. This study highlights the loss of genetic diversity in small isolated horse populations and the urgent need to take protective measures to preserve them.

Keywords: Indigenous Greek horses, Rare breeds, Microsatellites

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1. Introduction

Animal genetic resources make up part of natural and cultural world heritage. The loss of various distinct breeds or populations threatens the genetic variability and adaptation ability of domestic species. Hence, the study and preservation of rare indigenous horse populations is of great importance, both nationally and globally.

Islands of Aegean archipelagos and Crete, with their characteristic warm and dry climate, geographical isolation and rocky ground, are host to several indigenous horse populations that display unique properties and adaptations (Cothran et al., 2010; Bömcke et al., 2011; Kostaras et al., 2021). Most of these are now rare or nearly extinct, with their status classified as endangered or critical. They lack genetic characterization and a systematic preservation plan. Due to their small population size, further difficulties arise for their recognition as breeds and proper management. Modern genetic tools such as microsatellites can play an important role in identification and preservation of horse populations or breeds (Cothran and Luis, 2005; Van de

Goor et al., 2010).

Main documented characteristics of the studied horse populations include exceptional resilience to draught and heat, tolerance to poor nutrition, with physical and behavioral adaptations to fit the challenging mountainous ground and local conditions (from strong feet and small, hard hoofs to alert but self-composed temperament). They also exhibit low susceptibility to parasites, disease and physical injury, compared to other horses on the same environment (Giantsis et al., 2020; Kostaras et al., 2021). The oldest, traditional type has remarkably small body size and has been able to survive for long periods of time without human intervention in mountainous regions, forming feral populations (Papaioannou and Kostaras, 2006). The larger sized, gaiter type breeds are best suited for riding, but have higher maintenance demands. Greek "gaiters", have the ability to perform an extra, high-speed, two-beat lateral gait, also known as "pace", where both limbs from each side of the horse move simultaneously, due to a dominant nonsense mutation in DMRT3 gene affecting locomotion (Promerová et al, 2014).

Attempts have been conducted to study the horse populations in Greece using DNA markers such as RAPDs (Apostolidis et al., 2001), microsatellites (Cothran et al., 2010) or microsatellites and allozymes (Bömcke et al., 2011).

For this study, five indigenous horse populations were considered. Three of them are traditional miniature horses (Skyros Small Horse, Rodos Small Horse and Midili Small Horse) and two are larger gaiter types (Lesvos and Messara). From the above populations, only Skyros Small Horse and Messara horses are officially recognized as breeds and have studbooks (Centre of Animal Genetic Resources of Athens, 2019). This preliminary study of local Greek horse populations aims to support their conservation by determination of their genetic characterization. The objective is to study their genetic structure by defining the number of alleles, testing for Hardy-Weinberg equilibrium and estimating genetic diversity indices.

2. Materials and Methods

2.1. Animal Materials

The sampled horses were chosen according to previous breed/population descriptions and parentage information (Scherf, 2000; Kostaras et al., 2021). They form two distinct phenotypes: miniature, non-gaiting type with withers height (WH) ranging from 105-115 cm (Skyros Small Horse, Rodos Small Horse and Midili Small Horse) and larger gaiter type with a WH of 130 to 150 cm (Lesvos Gaiter and Messara breed). For this study, 46 hair root follicle samples were used (10 to 20 rooted hairs from each horse) from 5 local horse populations (Figure 1): Skyros Small Horse (n=9), Rodos Small Horse (n=6), Midili Small Horse (n=2), Lesvos Gaiter (n=22) and Messara breed (n=7). The Skyros Small Horse breed with around 160 purebred individuals is officially recognized as a breed and under protection (Centre of Animal Genetic Resources of Athens, 2019; Kostaras et al, 2021).

Figure 1. Phenotypic characteristics of 1) Skyros Small Horse breed, 2) Rodos Small Horse, 3) Midili Small Horse, 4) Messara breed and 5) Lesvos Gaiter.

Messara horse is a gaiting breed from the island of Crete, officially recognized by the state, with an estimated population of 100-200 horses (Centre of Animal Genetic Resources of Athens, 2019; Kostaras et al., 2021). Rodos Small Horse population consists of 10 reproductive horses from the homonymous island. Its population has passed through a recent severe bottleneck of 3 females and 3 males (Kostaras et al, 2021). Last feral herd was captured two decades ago and has been bred for preservation since then (Papaioannou and Kostaras, 2006). Rodos Small Horse horses are not recognized or protected by the state. Midili Small Horse population, from Lesvos Island, was considered extinct until recently when the presence of some small feral herds was reported (population size is estimated from 5 to 20 horses). The two sampled Midili horses are remains of an old population and the only unrelated individuals from two different feral herds, each representing a separate herd. Lastly, Lesvos Gaiter is not recognized as a breed by the state and its population size is unknown. The geographical location of the studied horses is shown in Figure 2.

Figure 2. geographical location of the studied horse populations.

The collected rooted hair was stored in plastic bags and submitted to Texas A&M University Animal Genetics Laboratory. DNA was isolated from the hair follicles using PUREGENE DNA purification kit according to the manufacturer's protocol and analyzed for 15 species-specific horse microsatellite loci (VHL20, HTG4, AHT4, HMS7, HTG6, AHT5, HMS6, ASB2, HTG10, HTG7, HMS3, HMS2, ASB17, ASB23 and LEX33) using multiplex PCR. Table 1 shows the analyzed microsatellite loci, their chromosome location and length in base pairs. Microsatellite analysis was achieved according to the procedures described by Juras et al. (2003), using the nomenclature of the International Society for Animal Genetics.

Table 1. Fifteen horse-specific microsatellite loci used in this study

Locus	Amplicon Length (bp)	Chromosome Location
VHL20	89-109	30
HTG4	127-141	9
AHT4	148-164	24
HMS7	173-187	1
HTG6	84-106	15
AHT5	130-146	8
HMS6	159-171	4
ASB2	222-254	15
HTG10	93-113	21
HTG7	120-130	4
HMS3	150-172	9
HMS2	284-304	10
ASB17	93-121	2
ASB23	183-217	3
LEX33	195-221	4

2.2. Statistical Analysis

Statistical analysis of the genotypic frequencies was conducted using the following programs: Create (Coombs et al, 2008), GENEPOP (Rousset, 2008), GeneAlEx (Peakall and Smouse, 2012) and GENETIX (Belkhir, 2004). We estimated allelic frequencies, number of alleles (N_a) and effective number of alleles (N_e) using GENETIX. Polymorphic information content (PIC) for each locus was calculated. We tested the Hardy-Weinberg equilibrium calculating F_{is} values in each locus with GENEPOP. Allelic richness (A_r) (El Mousadik and Petit, 1996) was calculated on the basis of the minimum sample size of 2 diploid individuals using HIERFSTAT (Goudet, 2005) in R (R core team, 2020). Estimators of genetic diversity included: the mean observed and expected heterozygosity [H_o, H_e (unbiased estimation according to Nei, (1978)], the mean genetic diversity within subpopulations (H_S), the total genetic diversity (H_T) and the proportion of genetic diversity that resides among populations (G_{ST}), which is equivalent of F_{ST}, calculated, applying the corrections of Nei and Chesser (1983) and Nei (1987) for small sized populations and inbreeding applied in the calculations of H_S and H_T. Furthermore, we applied three methods to investigate genetic structure of the studied populations: (i) the factorial correspondence analysis based upon allelic frequencies (GENETIX software), (ii) the population assignment method based upon genotypic frequencies of the studied populations according to Paetkau et al. (1995) and Paetkau et al. (2004) through GeneAlEx software and (iii) the metric multidimensional scaling (MDS) which was calculated with default distribution of R program (command cmdscaling; R core team, 2020) to project the multidimensional D_{PS} genetic distance matrix onto a two-dimensional (2D) plot for the 5 populations. The matrix of D_{PS} genetic distances between all pairs of individuals was estimated as D_{PS} = 1-PS. The proportion of shared alleles (PS) was calculated with Adegenet 2.0.0 (Jombart, 2008).

3. Results and Discussion

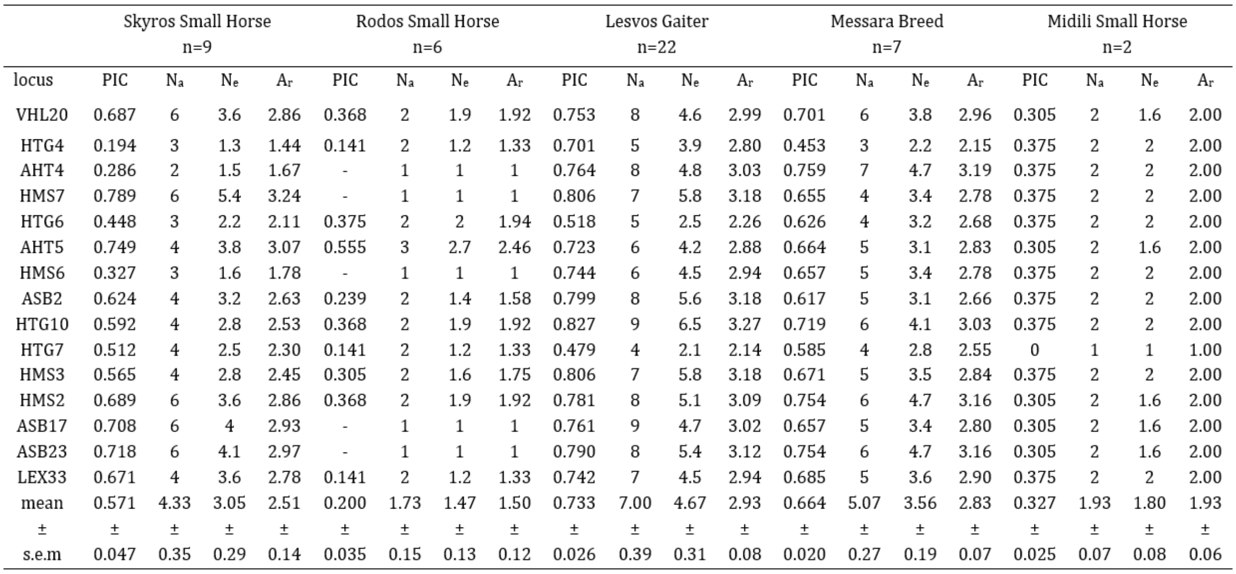
In total, 120 alleles were found across the 15 analyzed microsatellites and their allelic frequencies are presented in Supplementary Table 2. A minimum of four alleles was detected in HTG7 locus while a maximum of 13 alleles was found in ASB17 locus. The total number of alleles detected in each population was: 42 alleles for Skyros Small Horse, 20 alleles for Rodos Small Horse, 71 alleles for Lesvos Gaiter, 52 alleles for Messara breed and 21 alleles for Midili Small Horse (Supplementary Table 2). Genetic variability indices per locus and population are shown in Table 3. The mean number of alleles Na ranged from 1.73±0.15 (Rodos Small Horse) to 7.00±0.39 (Lesvos Gaiter). The effective number of alleles Ne ranged from 1.47±0.13 (Rodos Small Horse) to 4.67±0.31 (Lesvos Gaiter) and the PIC was between 0.200±0.035 (Rodos Small Horse) and 0.733±0.026 (Lesvos Gaiter). Because of the different sample sizes, we calculated allelic richness Ar, which ranged between 1.50 (Rodos Small horse) and 2.93 (Lesvos Gaiter). All estimators of genetic diversity reached highest values in Lesvos Gaiter in comparison with the other populations with the highest PIC (mean=0.733±0.026), N_e (mean=4.668±0.309) and A_r (mean=2.93±0.08). The Skyros Small Horse (mean PIC=0.571±0.047 and N_e=3.051±0.290 and Messara breed (mean PIC=0.664±0.020, N_e=3.559±0.186) had intermediate levels of genetic variability. The Rodos Small Horse had the lowest levels (mean PIC=0.200±0.035, N_e=1.469±0.134) and five monomorphic loci. The Midili Small Horse was excluded from further analysis due to inadequate sample number. The Skyros Small population showed slightly lower PIC values (0.571) than in 2011 (0.598) (Bömcke et al, 2011) but our sample was smaller.

As seen on Table 4, Hardy-Weinberg (H-W) equilibrium was not violated on most populations. Messara breed showed the highest number of loci that deviated from H-W equilibrium (ASB2, HTG10, LEX33) but sample size was low. The mean multilocus observed heterozygosity (H_o) was highest on Lesvos Gaiter population (0.764±0.0.027), followed by Messara breed (0.733±0.052), and then Skyros Small (0.689±0.0.051). The Rodos Small Horse had the lowest heterozygosity values as expected (0.300±0.075) due to its small size and population history. The Skyros Small Horse population from this study showed slightly higher heterozygosity values (expected heterozygosity He=0.656±0.049 and observed heterozygosity Ho=0.689±0.051) than in 2011 (0.621 and 0.647, respectively) (Bömcke et al., 2011) despite lower PIC values, which could possibly be due to sampling or analysis variation. It is worth mentioning that Bömcke et al. (2011) used nearly the same set of microsatellites as we did, except LEX33, plus they used HMS1 and CA425, and they had 99 Skyros samples. The above heterozygosity estimations are within the range of values reported in rare horse populations around the world. From the literature review examples given are the Japanese Tsushima (n=25; Ho=0.66), Kiso (n=55; Ho=0.67) and Tokara (n=110; Ho=0.44) breeds (Kakoi et al, 2007; Takasu et al, 2012) or the Knabstupper horse from Denmark (n=170; Ho=0.71) (Thirstrup et al., 2008) and other breeds (Scherf, 2000). In relation to the above, the Rodos Small Horse, with its extremely small and isolated population, had low heterozygosity estimations. F_{is} values (Table 5) showed that among the main four populations Rodos Small Horse had an excess of heterozygotes (F_{is} = -0.130), which is in contrast with the known history of genetic isolation, inbreeding and a severe bottleneck effect of this population. In many cases Fis index is affected by the extremely small sample size (<10) and from the fact that the sampled individuals Rodos Small Horse are all relatives.

G_{ST} values representing a multiallelic expansion of Wrights F_{ST} ranged between -0.004 (HTG6 locus) and 0.362 (HTG4 locus), indicating from low (less than 0.150) to strong (more than 0.250) differentiation among populations, depending on the locus. The average G_{ST} value among Skyros Small Horse, Rodos Small Horse, Lesvos Gaiter and Messara breed populations was high at 0.161, showing that 16% of the total genetic variability was explained by population differentiation. It is worth mentioning that this differentiation also has a geographical component as each of these populations come from different islands.

The factorial correspondence analysis based upon allelic frequencies (Figure 3), the population assignment diagram (Figure 4) and the metric multidimensional scaling (MDS) (Figure 5) showed the same pattern of distribution at the individual level. The above analyses reveal two clusters, with the individuals of Rodos Small Horse separated from the remaining populations. In the second cluster, the different colors for individuals of Lesvos, Messara and Midili are combined, while Skyros individuals within this cluster were more distinct. The above analyses confirm the distinctiveness and relative homogeneity of Rodos and Skyros Small Horses. On the other hand, Lesvos Gaiter and Messara breed, the populations with the highest genetic diversity estimates as previously noted, seem quite heterogeneous as their individuals are not separated from each other and occupy a common area on the plot (Figure 3). The clear differentiation between the Skyros Small Horse and Messara breed (Crete) populations found here, is in agreement with previous findings using RAPD DNA markers (Apostolidis et al., 2001). The two unique Midili Small Horse individuals seem related to Lesvos Gaiter individuals, not surprisingly, since they are from the same island. Using two different analyses the same two clusters are depicted in the population assignment and MDS diagrams (Figure 4 and Figure 5).

Table 3. Genetic variability estimations per locus and population



	Skyros Small Horse n=9				Rodos Small Horse n=6				Lesvos Gaiter n=22				Messara Breed n=7				Midili Small Horse n=2			
locus	PIC	N_a	N_e	A_r	PIC	N_a	N_e	A_r	PIC	N_a	N_e	A_r	PIC	N_a	N_e	A_r	PIC	N_a	N_e	A_r
VHL20	0.687	6	3.6	2.86	0.368	2	1.9	1.92	0.753	8	4.6	2.99	0.701	6	3.8	2.96	0.305	2	1.6	2.00
HTG4	0.194	3	1.3	1.44	0.141	2	1.2	1.33	0.701	5	3.9	2.80	0.453	3	2.2	2.15	0.375	2	2	2.00
AHT4	0.286	2	1.5	1.67	-	1	1	1	0.764	8	4.8	3.03	0.759	7	4.7	3.19	0.375	2	2	2.00
HMS7	0.789	6	5.4	3.24	-	1	1	1	0.806	7	5.8	3.18	0.655	4	3.4	2.78	0.375	2	2	2.00
HTG6	0.448	3	2.2	2.11	0.375	2	2	1.94	0.518	5	2.5	2.26	0.626	4	3.2	2.68	0.375	2	2	2.00
AHT5	0.749	4	3.8	3.07	0.555	3	2.7	2.46	0.723	6	4.2	2.88	0.664	5	3.1	2.83	0.305	2	1.6	2.00
HMS6	0.327	3	1.6	1.78	-	1	1	1	0.744	6	4.5	2.94	0.657	5	3.4	2.78	0.375	2	2	2.00
ASB2	0.624	4	3.2	2.63	0.239	2	1.4	1.58	0.799	8	5.6	3.18	0.617	5	3.1	2.66	0.375	2	2	2.00
HTG10	0.592	4	2.8	2.53	0.368	2	1.9	1.92	0.827	9	6.5	3.27	0.719	6	4.1	3.03	0.375	2	2	2.00
HTG7	0.512	4	2.5	2.30	0.141	2	1.2	1.33	0.479	4	2.1	2.14	0.585	4	2.8	2.55	0	1	1	1.00
HMS3	0.565	4	2.8	2.45	0.305	2	1.6	1.75	0.806	7	5.8	3.18	0.671	5	3.5	2.84	0.375	2	2	2.00
HMS2	0.689	6	3.6	2.86	0.368	2	1.9	1.92	0.781	8	5.1	3.09	0.754	6	4.7	3.16	0.305	2	1.6	2.00
ASB17	0.708	6	4	2.93	-	1	1	1	0.761	9	4.7	3.02	0.657	5	3.4	2.80	0.305	2	1.6	2.00
ASB23	0.718	6	4.1	2.97	-	1	1	1	0.790	8	5.4	3.12	0.754	6	4.7	3.16	0.305	2	1.6	2.00
LEX33	0.671	4	3.6	2.78	0.141	2	1.2	1.33	0.742	7	4.5	2.94	0.685	5	3.6	2.90	0.375	2	2	2.00
mean	0.571	4.33	3.05	2.51	0.200	1.73	1.47	1.50	0.733	7.00	4.67	2.93	0.664	5.07	3.56	2.83	0.327	1.93	1.80	1.93
±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
s.e.m	0.047	0.35	0.29	0.14	0.035	0.15	0.13	0.12	0.026	0.39	0.31	0.08	0.020	0.27	0.19	0.07	0.025	0.07	0.08	0.06

PIC= polymorphism information content, Na= number of alleles, Ne= effective number of alleles, Ar= allelic richness.
“-“= the PIC value in monomorphic loci. s.e.m= standard error of the mean

Table 4. Genetic diversity estimations per locus and population. (expected heterozygosity He, observed heterozygosity Ho, diversity within subpopulations HS, total diversity HT, diversity that resides between subpopulations GST)

Locus	H	Skyros Small Horse n=9	Rodos Small Horse n=6	Lesvos Gaiter n=22	Messara Breed n=7	Genetic diversity per locus (total estimates)		
						H_S	H_T	G_{ST}
VHL20	H_e	0.765	0.530	0.801	0.791	0.721	0.831	0.132
	H_o	0.667	0.833	0.818	0.714			
HTG4	H_e	0.216	0.167	0.760	0.582	0.435	0.681	0.362
	H_o	0.222	0.167	0.818	0.571			
AHT4	H_e	0.366	0	0.810	0.846	0.508	0.726	0.301
	H_o	0.444	0	0.773	1			
HMS7	H_e	0.863	0	0.848	0.758	0.620	0.786	0.211
	H_o	0.889	0	0.909	0.857			
HTG6	H_e	0.582	0.546	0.610	0.736	0.612	0.610	-0.004
	H_o	0.778	0.667	0.545	0.857			
AHT5	H_e	0.830	0.682	0.778	0.747	0.760	0.821	0.075
	H_o	0.778	0.667	0.773	0.857			
HMS6	H_e	0.386	0	0.797	0.758	0.492	0.583	0.157
	H_o	0.444	0	0.727	0.714			
ASB2	H_e	0.726	0.303	0.841	0.725*	0.660	0.775	0.148
	H_o	0.667	0.333	0.864	0.286			
HTG10	H_e	0.686	0.530	0.865	0.813*	0.721	0.797	0.092
	H_o	0.667	0.500	0.864	1			
HTG7	H_e	0.628	0.167	0.532	0.692	0.503	0.665	0.243
	H_o	0.667	0.167	0.546	0.857			
HMS3	H_e	0.673	0.409	0.848	0.769	0.681	0.797	0.146
	H_o	0.667	0.500	0.818	0.571			
HMS2	H_e	0.765	0.530	0.825	0.846	0.748	0.774	0.033
	H_o	0.778	0.500	0.682	0.714			
ASB17	H_e	0.791	0	0.808	0.758	0.591	0.750	0.213
	H_o	1	0	0.773	0.857			
ASB23	H_e	0.797	0	0.833*	0.846	0.629	0.780	0.193
	H_o	0.778	0	0.727	0.714			
LEX33	H_e	0.765	0.167	0.794	0.780*	0.634	0.734	0.136
	H_o	0.889	0.167	0.818	0.429			
Multi-locus (mean±s.e.m)	H_e	0.656±0.049	0.269 ±0.064	0.783±0.024	0.763±0.023	0.621 ±0.026	0.741 ±0.019	0.161 ±0.025
	H_o	0.689±0.051	0.300±0.075	0.764±0.027	0.733±0.052			

*The significant differences between H_e and H_o, s.e.m= standard error of the mean

Table 5. Fis values by locus and population*

Locus	Skyros Small Horse	Rodos Small Horse	Lesvos Gaiter	Messara Breed
VHL20	0.195	-0.667	-0.022	0.104
HTG4	-0.032	-0.000	-0.078	0.020
AHT4	-0.231	-	0.047	-0.200
HMS7	-0.032	-	-0.074	-0.143
HTG6	-0.366	-0.250	0.108	-0.180
AHT5	0.067	0.024	0.007	-0.161
HMS6	-0.164	-	0.089	0.062
ASB2	0.086	-0.111	-0.027	0.625
HTG10	0.030	0.063	0.001	-0.254
HTG7	-0.067	-0.000	-0.026	-0.263
HMS3	0.010	-0.250	0.036	0.273
HMS2	-0.018	0.063	0.176	0.167
ASB17	-0.286	-	0.044	-0.143
ASB23	0.026	-	0.130	0.167
LEX33	-0.174	-0.000	-0.031	0.471
mean	-0.054	-0.130	0.026	0.042

*Fis value="-" in monomorphic loci. (Midili was excluded)

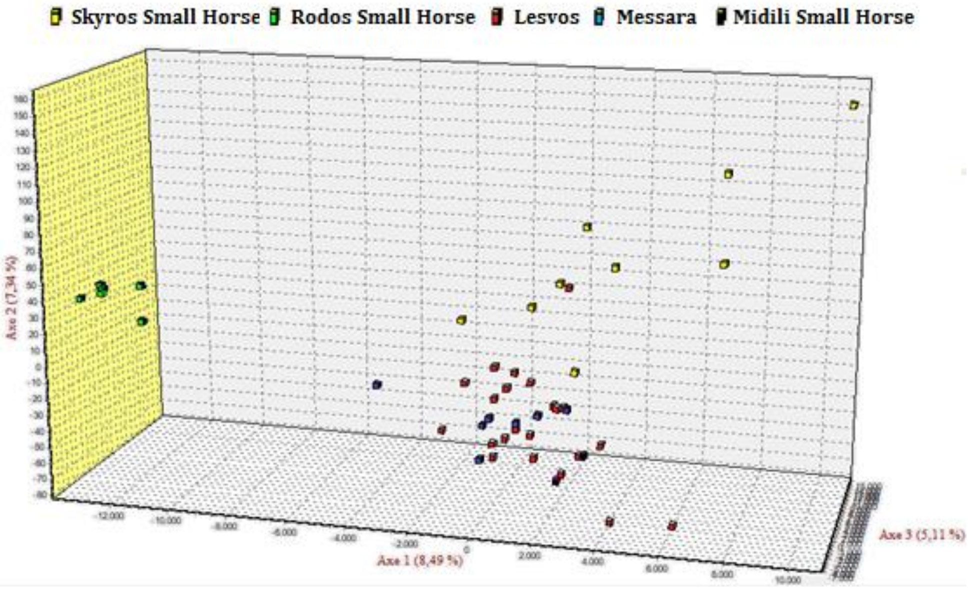


Figure 3. Diagram of factorial correspondence analysis of the studied horses.

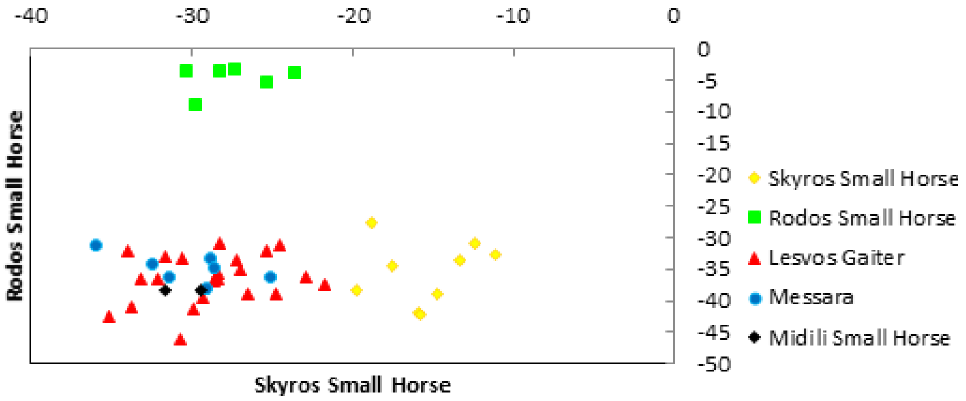


Figure 4. Population assignment diagram of the studied horses.

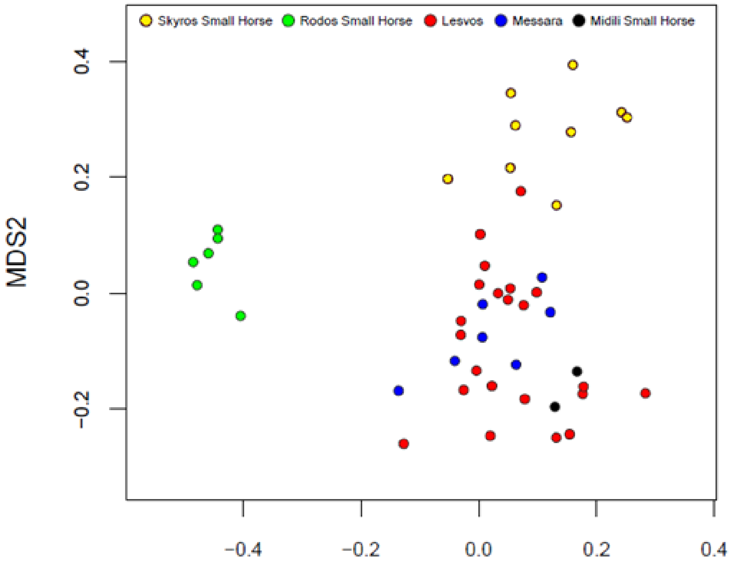


Figure 5. Diagram of metric multidimensional scaling (MDS) analysis of the studied horses.

It is worth mentioning that miniature horses like the Skyros Small (probably the most studied Greek breed) (Apostolidis et al., 2001; Bömcke et al., 2011; Laliotis and Avdi, 2017), Rodos Small and Midili Small were abundant on the islands until two centuries ago. Then they were practically abandoned after the onset of larger breeds for riding and their use as working animals was reduced. The surviving herds were then discarded and their numbers declined significantly. Although almost extinct now, these horses seem to have contributed significantly to modern Greek horse populations. Lesvos Gaiter for example seems to have evolved from crossings between the local Midili Small Horse and Greek or foreign gaiters. On the other hand, Lesvos and Messara gaiters are much appreciated by locals, who favor their gait and cross them in an effort to preserve them by their own initiative, without any specific, organized plan of mating. The above horse populations are often confused with each other and they are crossed with other gaiting breeds of Greek or foreign origin, a practice that puts efforts to preserve and establish them as breeds at risk while eroding their genetic character.

Summarizing our findings, Lesvos Gaiter population showed sufficiently large amounts of genetic variability and this likely is due to its heterogeneous origin. It is suggested that a registry for the Lesvos be set up with clearly defined breed standards. Removal of individuals that do not meet these standards will help create uniformity within the population but it is important that genetic diversity be maintained to the greatest degree possible. Avoiding crosses with other breeds is a necessity. The Messara breed and Skyros Small Horse had intermediate levels of heterozygosity and high PIC values relative to their medium low population size and are therefore possibly not at immediate risk of genetic erosion and extinction. Finally, Rodos Small Horse and Midili Small Horse are at the verge of extinction with population sizes of less than 20 individuals and largely inbred, as suggested by our findings. The two populations were found to have low levels of genetic variability that would possibly still allow their survival if conservation efforts were to be urgently fortified, focusing on controlled breeding practices to avoid any further loss of variability.

In general, the insufficient individual ID tracking (by microchip or other) of the animals and lack of pedigrees (except Skyros Small Horses) in Greece are prominent problems that strongly affect the efforts of protection of indigenous breeds. The onset of widely acceptable, objective breed standards and further study of the Greek breeds would be of great importance for their official recognition and preservation.

4. Conclusion

From our analysis, the estimations of genetic variability showed promising levels for Lesvos Gaiter, Messara Breed and Skyros Small Horses that seem to have good chances of preservation if properly managed. Rodos Small Horse and Midili Small Horse are clearly in danger of extinction and only well-planned and immediate protective measures could rescue them.

In the future, we plan to add more samples from these and other Greek horses as well as foreign breeds to our dataset in order to have a better depiction of the genetic relationships among these populations.

Author Contributions

The percentage of the author(s) contributions is present below. All authors reviewed and approved final version of the manuscript.

	M.E.K	D.P.	N.K.	G.P.L.	I.B.	E.G.C.	R.J.	P.K.
C			34		33			33
D			25		25	25		25
S			25			25	25	25
DCP	20		20	20	20		20	
DAI	34	33						33
L	25	25		25				25
W	50							50
CR				20	20	20	20	20
SR	50				25			25
PM					25	25	25	25
FA			25		25	25	25	

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

All experimental procedures were approved by the Bioethical Committee of the Agricultural University of Athens under the guidelines of Council Directive regarding the protection of animals used for experimental and other scientific purposes (protocol code: 86/609/EEC and date of approval: April 14, 2021).

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References

Apostolidis AP, Mamuris Z, Karkavelia E, Alifakiotis T. 2001. Comparison of Greek breeds of horses using RAPD markers. J Anim Breed Genet, 118(1): 47-56.

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F. 2004. GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier, France.

Bömcke E, Gengler N, Cothran EG. 2011. Genetic variability in the Skyros pony and its relationship with other Greek and foreign horse breeds. Genet Mol Biol, 34: 68-76.

Centre of Animal Genetic Resources of Athens. 2019. URL: http://www.kgbzath.gr (access date: August 10, 2022).

Coombs JA, Letcher BH, Nislow KH. 2008. Create: a software to create input files from diploid genotypic data for 52 genetic software programs. Mol Ecol Resour, 8: 578-580. DOI: 10.1111/j.1471-8286.2007.02036.x.

Cothran EG, Luis C. 2005. Genetic distance as a tool in the conservation of rare horse breeds. Pub-Eur Assoc Anim Prod, 116: 55.

Cothran G, Kostaras N, Juras, R, Conant E. 2010. Genetic diversity in native Greek horses. In: Book of Abstracts of the 61st Annual Meeting of the European Association for Animal Production, August 23-27, Heraklion, Greece, pp: 210.

El Mousadik A, Petit RJ. 1996. High level of genetic differentiation for allelic richness among populations of the argan tree [Argania spinosa (L.) Skeels] endemic to Morocco. Theoret. Appl Genet, 92: 832-839. DOI: 10.1007/BF00221895.

Giantsis IA, Diakakis NE, Avdi M. 2020. High frequencies of TNC and COL5A1 genotypes associated with low risk for superficial digital flexor tendinopathy in Greek indigenous horse breeds compared with warmblood horses. J Equine Vet Sci, 92: 103173.

Goudet J. 2005. HIERFSTAT, a package for R to compute and test hierarchical F-statistics. Mol Ecol Notes, 5: 184-186.

Jombart T. 2008. adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics, 24: 1403-1405.

Juras R, Cothran EG, Klimas R. 2003. Genetic analysis of three Lithuanian native horse breeds. Acta Agric Scand (A), 53(4): 180-185.

Kakoi H, Tozaki T, Gawahara H. 2007. Molecular analysis using mitochondrial DNA and microsatellites to infer the formation process of Japanese native horse populations. Biochem Genet, 45(3): 375-395.

Kostaras N, Papaioannou K, Konstantinidou M. 2021. Greek equine breeds in “Greek Domestic Breeds, A hidden treasure”. AMALTHIA - Network for the protection of Greek indigenous farm animals, Athens, Greece, 1st ed, pp: 152.

Laliotis GP, Avdi M. 2017. Genetic diversity assessment of an indigenous horse population of Greece. Biotech Anim Husb, 33(1): 81-90.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89(3): 583-590.

Nei M. 1987. Phylogenetic trees in molecular evolutionary genetics. Annu Rev Genet, 30: 371-403. DOI: 10.1146/annurev.genet.30.1.371.

Nei M, Chesser RK. 1983. Estimation of fixation indices and gene diversities. Annals Human Genet, 47(3): 253-259.

Paetkau D, Calvert W, Stirling I, Strobeck C. 1995. Microsatellite analysis of population structure in Canadian polar bears. Mol Ecol, 4(3): 347-354.

Paetkau D, Slade R, Burden M, Estoup A. 2004. Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. Mol Ecol, 13(1): 55-65.

Papaioannou K, Kostaras N. 2006. Study of the indigenous horse breed of Rhodes, in Greek. AMALTHIA -Network for the P rotection of Greek Indigenous Farm Animals, Athens, Greece, 1st ed, pp: 152.

Peakall R, Smouse PE. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics, 28: 2537-2539.

Promerová M, Andersson LS, Juras R, Penedo MCT, Reissmann M, Tozaki T, Andersson L. 2014. Worldwide frequency distribution of the ‘Gait keeper’mutation in the DMRT 3 gene. Anim Genet, 45(2): 274-282.

R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/ (access date: September 10, 2021).

Rousset F. 2008. Genepop’007: A complete reimplementation of the Genepop. Mol Ecol Resour, 8(1): 103-106.

Scherf BD. 2000. World watch list of domestic animal diversity.

Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, 3rd ed., pp: 37.

Takasu M, Hiramatsu N, Tozaki T, Kakoi H, Nakagawa T, Hasegawa T, Maeda HM, Murase T, Mukoyama H. 2012. Genetic characterization of the endangered Kiso horse using 31 microsatellite DNAs. J Vet Medic Sci, 74(2): 161-166.

Thirstrup JP, Pertoldi C, Loeschcke V. 2008. Genetic analysis, breed assignment and conservation priorities of three native Danish horse breeds. Anim Genet, 39(5): 496-505.

Van de Goor L, Panneman H, van Haeringen W. 2010. A proposal for standardization in forensic equine DNA typing: allele nomenclature for 17 equine-specific STR loci. Anim Genet, 41(2): 122-127.