

# GENETIC DIVERSITY ASSESSMENT OF AN INDIGENOUS HORSE POPULATION OF GREECE

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Original scientific paper

**Abstract:** Highly endangered local breeds are considered important not only for the maintenance of their genetic diversity for future survival but also because they regarded as part of the cultural heritage of the local and national communities. Using pedigree data and an analysis of 18 microsatellite loci we investigated the genetic diversity of a private (commercial) indigenous Skyros horse population, reared in an insular region of North-Western of Greece. The overall average animal inbreeding value reached 24%. Concerning average inbreeding value over non founding animals, it was estimated to 0.013, while the corresponding value over inbred animals were 0.13. The mean number of alleles per locus amounted to 3.72, ranging between 1 and 7 alleles. The average observed heterozygosity was 0.57. Taking into account the inbreeding estimated index, an average heterozygote deficit ( $F_{is}$ ) of -0.09 was noted ( $P < 0.05$ ). Although the population maintained reasonable levels of genetic diversity, well studied inbreeding strategies should be implemented, in order to reduce the loss of genetic variability, to avoid extinction and further genetic drift of the population.

**Keywords:** Skyros Horse, Inbreeding, Conservation, STRs, genetic markers.

## Introduction

Many local indigenous breeds of domestic animals (i.e. horse, sheep) are considered highly endangered due to their small population number. The proper genetic management of such populations is crucial for their survival and it involves two major steps. The first step involves the selection of the individuals who will be permitted to leave descendants and the second the design of the proper mating scheme. However, in order the aforementioned steps to be implemented, the

genetic diversity (inbreeding and heterozygosity levels) has primary to been considered.

High inbreeding and low heterozygosity levels are associated with undesired characteristics i.e. reduce fitness, reproductive capacity and survival (Falconer MacKay, 1996). Such characteristics are often observed in small populations of endangered breeds. The conservation of these breeds is justifiable due to their importance in their local environmental conditions.

Skyros horse, a small-sized pony originated from the homonymous Greek island, belongs to the highly endangered breeds in Greece rendering its population under evaluation regularly. Currently, less than 140 purebred Skyros horses have been recorded throughout the country and phenotypic characteristics have been well documented (SAVE, 2015). The fact that the majority of these horses roam freely and well-designed breeding schemes are missing renders the future of the breed under severe challenge.

The implementation of novel DNA techniques, such as microsatellite molecular markers (STRs), in combination with data retrieved from pedigree analysis render the identification of genetic diversity a very useful and precise tool for further breeding conservation strategies (Marleta et al., 2006; Criscione et al., 2015). Previous analysis of an experimental population of Skyros pony showed low levels of genetic diversity (Avdi and Banos, 2008). As inbreeding leads to undesired performance characteristics investigating the genetic background of Skyros horses is always of great importance as it would increase the chances for survival. The aim of the present study was to examine the genetic diversity of a small population of Skyros horses, reared in an insular region of North-Western Greece, away from its natural origin habitat. To the best of our knowledge this is the first time that the certain population is thoroughly analyzed presenting the most recent assessment of the genetic diversity levels, which may assist the future survival of the breed.

## Materials and Methods

### *Population data*

Data were retrieved from a private commercial farm of Skyros horses reared in an insular region of North-Western Greece (Corfu). A total number of 25 purebred animals (18 females and 7 males) with pedigree data were taken into account. Mating was at random.

A genealogical tree was developed using animal pedigrees and thereafter inbreeding coefficients for each animal were calculated based on the genetic relationships among their parents. Inbreeding levels of common ancestors were also taken into account. Concerning founder animals (without own pedigree), a total of 9 animals (6 females and 3 males) did not have any ancestor information. However, these were considered to be among the population founding animals that

were unrelated to each other. Inbreeding levels in the studied population were assessed based on the calculated genetic relationships among animals and according to F-statistic (Wright, 1978) as computed by Caballero and Toro (2002).

### ***STRs marker genotyping***

A set of 18 microsatellite markers were genotyped in the present study. Genomic DNA was firstly isolated from blood and then the desired STR sequence was amplified using the Polymerase Chain Reaction (PCR) and analysed at the premises of Labogena in France as previously described (Avdi and Banos, 2008). Allelic discrimination was conducted using GeneMapper Software (Applied Biosystems). The guidelines proposed by the International Society of Animal Genetics (Solis *et al.*, 2005) were followed for the nomenclature of each marker.

### ***Statistical analysis***

The following parameters were determined for each marker: i) number and frequency of alleles, ii) observed heterozygosity ( $H_o$ ), iii) expected heterozygosity ( $H_e$ ), iv) heterozygote deficit ( $F_{is}$ ), v) Hardy-Weinberg deviation vi) Polymorphism Information Content (PIC), vii) probability of identity ( $P_i$ ), and viii) probability of parental exclusion ( $P_e$ ) (considering 1 or 2 parents). Allelic frequency was estimated by direct counting.

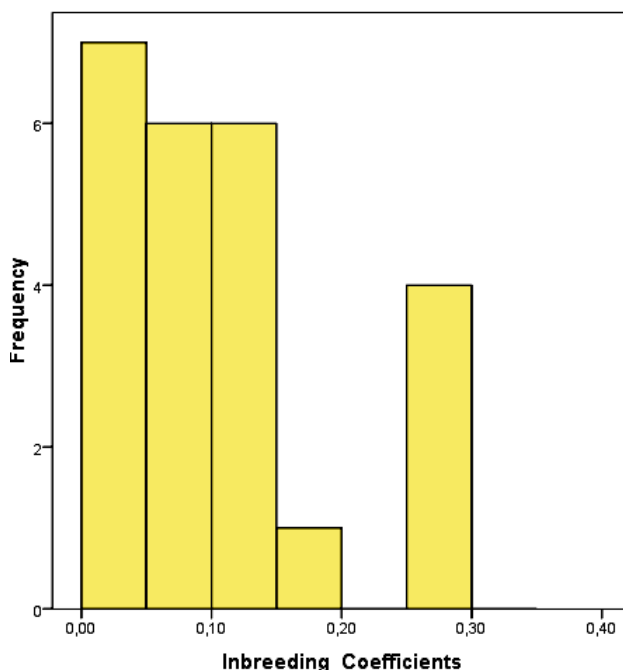
The parameters concerning heterozygosity (observed/expected) and heterozygote deficit were calculated using POPGENE software (Yeh *et al.*, 1999). Deviations from Hardy-Weinberg equilibrium were calculated using GENEPOP software (Raymond and Rousset, 1995), while the on-line platform at <http://w3.georgikon.hu/pic/english/kezi.aspx> was used to calculate Polymorphism Information Content (PIC) on the basis of observed allele frequencies (Botstein *et al.*, 1980). IDENTITY software was used in order to calculate the probability of identity for each marker ( $P_i$ ) and the probability of parental exclusion ( $P_e$ ) for each marker separately and across all 18 markers respectively. Within inbreeding population estimates ( $F_{is}$ ) was determined using FSTAT software (Goudet, 1995).

## **Results and Discussion**

Genetic diversity in farmed animals is of utmost importance in order: a) to meet current production needs in various environments, b) to allow sustained genetic improvement and c) to facilitate rapid adaptation to changing (Notter, 1999). In endangered populations (i.e. Skyros pony), genetic characterization and avoidance of inbreeding becomes the first crucial and necessary step for breed conservation and application of future breeding strategies.

***Pedigrees data: Intra-breed diversity***

According to our–data, an 18.8% (3 animals) of the 16 non-founding animals were found to be inbred in the population. The overall average animal inbreeding value reached the 24%. Concerning the average inbreeding value over non founding animals, it was estimated to 0.013, while the corresponding value over inbred animals were 0.13. Inbreed coefficients frequency across all inbred animals was ranged between 0.0234 and 0.30 (Fig.1). No statistical significant differences were noted between males and females ( $P>0.05$ ). Similar inbreeding profiles have been reported by previous author concerning other horses' small populations (*Curik et al., 2003; Aberle et al., 2004; Valera et al., 2005; van Eldik et al., 2006*). Interestingly, according to *Avdi and Banos (2008)* who analysed using pedigree data the genetic structure of a Skyros pony population, reared in an experimental university farm, revealed higher levels of inbreeding. Specifically, an extra 13% of the animals (32%) were found to be inbred in the total population and the average inbreeding value over non founding animals estimated to 0.03, revealing by means of genetic drift a narrow genetic status of the experimental population.



**Figure 1.** Frequency of inbreeding coefficients in the examined population populations

### ***Genetic variability of molecular markers***

Almost all the analysed microsatellite loci were found to be polymorphic apart from HTG4. The number of alleles ranged from 2 (HMS1) to 7 (ASB17) with a mean of 3.72 per locus, respectively (Table 1). The average allele frequency was estimated to 0.27.

Polymorphism Information Content (PIC) refers to the value of a marker for detecting polymorphism within a population. It depends on the number of detectable alleles and the distribution of their frequency. According to Botstein et al., 1980, when  $PIC > 0.5$  the marker is considered as highly informative while if  $(0.5 > PIC > 0.25)$  or  $(PIC < 0.25)$  the marker is considered as reasonably informative or slightly informative, respectively. In regard to the PIC in the examined population, the majority of loci were highly informative ( $PIC > 0.5$ ), while the rest were reasonable informative ( $0.5 > PIC > 0.25$ ) The observed mean number of alleles per microsatellite loci was closer to the estimates reported for inbred Basques horses (4.33 per locus) (Solis et al., 2005) or other European horses (4.50) (Luis et al., 2007) and lower to that noted for South American (5.67-7.67), North American (6-7.25), Caspian (8.69), an Italian (9.58) and a local Brazilian (14.36) horses breed (Luis et al., 2007; Pieragostini et al., 2005; Shasavarani and Rahimi-Mianji, 2010; Silva et al., 2012). The differences in average number of observed alleles may be attributed to different set of microsatellite markers, number of markers, population structure (i.e. divided into folks) and different horse breed (i.e. local Brazilian horses, Caspian horses, Pindos pony etc.).

Concerning the observed heterozygosity, it ranged from 0.19 to 0.90 with a mean of 0.57 (Table 1). Similar levels of heterozygosity have been reported in many other international horses' breeds i.e. North American horses (Colling and Kelly, 1996), Kladruber horse (Horin et al., 1998), Lipizzan horses (Curik et al., 2003), Norwegian horse's breeds (Bjørnstad et al., 2000), Sorraia horse (Luis et al., 2007) and other European horses (Solis et al., 2005). Further, taking into account the expected heterozygosity (Table 1), an average heterozygote deficit ( $F_{is}$ ) of -0.09 was estimated ( $P < 0.05$ ). Moreover, almost all loci were found in a Hardy-Weinberg equilibrium (Table 1) apart from loci LEX3 ( $P < 0.05$ ). The observed negative heterozygote deficit is indicative of a heterozygote surplus, meaning that more heterozygotes were observed than expected from allelic frequencies. It, also, suggests a potential outbreeding (i.e. occasional importation of external breeding animals into each population). Moreover, the lower average  $H_o$  compared to the  $H_e$  may reflect the narrow genetic base of the examined populations (Silva et al., 2012).

**Table 1. Measures of genetic diversity per analysed locus**

Marker	Number of alleles	Range of allele frequency	Theoretical Heterozygosity (He)	Observed Heterozygosity (Ho)	Heterozygote deficit (F <sub>is</sub> )	Polymorphism Information Content (PIC)	Hardy Weinberg Deviation (P value)
HTG6	3	0.19-0.57	0.5805	0.7143	-0.2305	0.52	0.4819
VHL20	4	0.14-0.43	0.70	0.81	-0.15	0.65	0.69
HTG10	3	0.10-0.74	0.42	0.52	-0.25	0.39	0.78
HTG4	1	-	-	-	-	-	
AHT5	5	0.07-0.62	0.66	0.71	-0.08	0.60	0.42
AHT4	3	0.17-0.62	0.54	0.67	-0.23	0.48	0.74
HMS3	5	0.07-0.60	0.60	0.67	-0.11	0.58	0.95
HMS6	2	0.21-0.79	0.34	0.33	0.01	0.27	1.00
HMS7	5	0.05-0.36	0.74	0.90	-0.23	0.70	0.167
HMS1	2	0.10-0.90	0.17	0.19	-0.11	0.16	1.000
ASB2	4	0.10-0.50	0.64	0.71	-0.11	0.60	0.917
ASB17	7	0.03-0.25	0.82	0.94	-0.15	0.79	0.537
ASB23	4	0.07-0.50	0.61	0.33	0.45	0.55	0.877
CA425	4	0.04-0.46	0.56	0.64	-0.13	0.48	0.214
HTG3	6	0.05-0.45	0.71	0.76	-0.07	0.69	0.860
HTG7	3	0.10-0.52	0.57	0.61	-0.08	0.55	0.313
LEX3	3	0.02-0.67	0.46	0.33	0.27	0.37	0.017*
LEX33	3	0.19-0.50	0.62	0.77	-0.24	0.55	0.720
Mean	3.72	0.27	0.57	0.63	-0.09	0.53	-
S.E.	0.12	0.03	0.04	0.05	0.04	0.48	-

\*P&lt;0.05

The probability of identity (P<sub>i</sub>) across all analysed markers was estimated at  $7.55 \times 10^{-12}$  reaching an extremely low level. This reflects to the fact that the probability of randomly selecting two animals with exactly the same genotypes in all markers is practically zero. Moreover, the overall probability of parental exclusion (P<sub>e</sub>) across all markers with 1 and 2 parents unconfirmed was 0.9798 and 0.9999, respectively, suggesting that the analyzed markers can be a useful tool for parentage verification in the population.

**Table 2. Comparison of genetic parameters in previously reported populations of Skyros horses.**

Population	N	He	Ho	F <sub>is</sub>	TNA	PIC
Present Study (whole reared examined population)	25	0.57	0.63	-0.06	3.72	0.53
Avdi and Banos (2008) (experimental population)	77	0.66	0.66	-0.09	4.11	0.58
Bomche et al. (2010) (joint sample analysis from different populations)	99	0.64	0.65	-0.005	5.93	0.59

\*N=population number; He: Expected Heterozygosity; Ho Observed Heterozygosity; Fis: Heterozygosity deficit; TNA=average number of alleles; PIC: polymorphism information content.

A few studies using STRs data have previously been reported in regard with the genetic structure of Skyros horse's population (*Criscione et al., 2015; Bomke et al., 2010*). In all reported populations a high heterozygosity excesses (Ho) was observed (Table 2) reaching similar levels to our results. In addition, an increase heterozygote deficit (F<sub>is</sub>) was noted for the population analysed by *Avdi and Banos (2008)* compared to the population analysed herein. Although the population analysed by *Bomke et al. (2010)* appeared to have lower inbreeding index (F<sub>is</sub>), it should be noted that this outcome results from a joint analysis of samples random chosen from three different subpopulations.

## Conclusions

The genetic structure of a whole Skyros horse population reared in an insular region of North-Western Greece was analysed presenting recent levels of genetic diversity. Our results showed that the population maintained considerably levels of genetic diversity despite its small census breeding environment compared to previous experimental population. A heterozygote excess from that expected as a result, probably, of outbreeding strategies were noted in the analysed population. All analysed genetic variability measures are comparable to results from other breeds. Taking into account that Skyros horse belongs to the endangered breeds, well studied inbreeding population strategies (i.e. avoid mating between relatives, equal participation of all parents to the next generation) should be implemented, in order to reduce the loss of genetic variability, to avoid extinction and further genetic drift of the whole population. Correlation between important traits (i.e. reproduction performance) and genetic diversity of the studied populations would be a further step aiming to assist the chances of breed's survival. The

simultaneously determination of the effective size of the whole population of Skyros horse, as a mean of genetic stabilization it would be undoubtedly a future challenge.

## **Procena genetičkog diverziteta autohtone populacije konja u Grčkoj**

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### **Rezime**

Visoko ugrožene lokalne rasa se smatraju važnim ne samo za održavanje njihove genetske raznolikosti za budući opstanak, već i zbog toga što se smatraju delom kulturne baštine lokalnih i nacionalnih zajednica. Koristeći podatke iz pedigre i analizu položaja 18 mikrosatelita, ispitivali smo genetsku raznolikost komercijalne autohtone populacije konja - Skyros, odgajane u ostrvskom regionu severozapadne Grčke. Ukupna prosečna inbriding vrednost dostigla je 24%. Srednji broj alela po lokusu iznosio je 3,72, u rasponu između 1 i 7 alela. Prosečna utvrđena heterozigotnost je bila 0,57. Uzimajući u obzir inbreeding indeks, prosečni heterozigotni deficit ( $F_{is}$ ) od -0,09 je utvrđen ( $P < 0,05$ ). Iako je populacija održala razumni nivo genetske raznovrsnosti, dobro proučena inbreeding strategija bi trebalo da se sprovodi, kako bi se smanjio gubitak genetske varijabilnosti, i izbegao nestanak i dalje genetski drift populacije.

**Ključne reči:** Skyros konji, inbriding, konzervacija, STR, genetski markeri

### **Acknowledgements**

The authors would like to thank Mrs Steen Sylvia, owner of the commercial farm, for her valuable cooperation. We would also thank Jean-Claude Meriaux and Jean-Michel Allamellou (Labogena, France) for their support in animal genotyping. Moreover, Professor George Banos is acknowledged for his useful comments on the manuscript and for the support on the statistical analysis. The Greek Ministry of Environment and the Greek Equestrian Club "Filippos Enois" partially funded the present study.



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