



Genetic diversity in Tunisian horse breeds

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Abstract. This study aimed at screening genetic diversity and differentiation in four horse breeds raised in Tunisia, the Barb, Arab-Barb, Arabian, and English Thoroughbred breeds. A total of 200 blood samples (50 for each breed) were collected from the jugular veins of animals, and genomic DNA was extracted. The analysis of the genetic structure was carried out using a panel of 16 microsatellite loci. Results showed that all studied microsatellite markers were highly polymorphic in all breeds. Overall, a total of 147 alleles were detected using the 16 microsatellite loci. The average number of alleles per locus was 7.52 (0.49), 7.35 (0.54), 6.3 (0.44), and 6 (0.38) for the Arab-Barb, Barb, Arabian, and English Thoroughbred breeds, respectively. The observed heterozygosities ranged from 0.63 (0.03) in the English Thoroughbred to 0.72 in the Arab-Barb breeds, whereas the expected heterozygosities were between 0.68 (0.02) in the English Thoroughbred and 0.73 in the Barb breeds. All F_{ST} values calculated by pairwise breed combinations were significantly different from zero ($p < 0.05$) and an important genetic differentiation among breeds was revealed. Genetic distances, the factorial correspondence, and principal coordinate analyses showed that the important amount of genetic variation was within population. These results may facilitate conservation programs for the studied breeds and enhance preserve their genetic diversity.

1 Introduction

Biological diversity or biodiversity refers to variability in the hereditary characteristics in a species. The highest genetic diversity level, for a species or a population, offers the opportunity for animals to challenge harsh environmental conditions and cope over with climate change and global warming. The worldwide breeds or populations' sizes went through continuous fluctuations that mirror environmental variation and demographic population growth (Willi et al., 2006; Andrew et al., 2011).

Recent statistics of the Food and Agriculture Organization (FAO) reported that the populations of numerous domestic animals, especially horses, are in steady decline, with some already extinct, thereby affecting both interbreed (decline in the actual number of equine breeds themselves) and

intra-breed (decline in the number of individuals) diversities (FAO, 2011). Horses' population sizes of the Tunisian breeds account for around 26 000 heads, of which 14 000, 6000, 5000, and 1000 are Arab-Barb, Barb, Arabian, and English Thoroughbred breeds, respectively. There are also 40 000 male and female mules (FNARC, 2015). These animals are generally used in the fantasia (traditional exhibition of horsemanship performed during cultural festivals), as well as in the equestrian sports. Appropriate conservation and sustainable management programs for the Tunisian equine breeds need comprehensive information about their genetic diversity and populations' structures. Concurrently, a high genetic diversity may indicate a genetic diversity hot spot, a tool for targeting conservation efforts in livestock species (Freeman et al., 2006; Khanshour et al., 2013).

Native horse breeds in Tunisia have been playing key roles through history in agriculture, transportation, and leisure activities. These breeds are now facing major constraints due to harsh climatic conditions and poor management practices. Haddad (2015) reported that the first studbook for Tunisian equine breeds was established in 20 June 1896. There were a few studies on the genetic diversity in the Tunisian horse breeds. The first study aimed at assessing autochthonous Tunisian horse's genetic diversity and was published in 2014 (Haddad et al., 2014; Jemmali et al., 2015). These authors attempted to characterize genetic relationships within and among local breeds (Barb, Arab-Barb and Mogod Pony). A poor genetic diversity was revealed. In the present time, there are attempts to start breeding programs for horses. Pedigree and performance recording are underway. Artificial insemination is a common practice in HARAS of FNARC in the north of Tunisia. Cross breeding is also used to upgrade breeds and to satisfy breeders' demand. There are, however, no conservation programs for equine breeds.

Microsatellite markers have been widely used to assess genetic variability for different horse breeds (Karima et al., 2011). Genetic diversity studies within and among horse breeds analyzed using microsatellites have been conducted on French breeds (Moureaux et al., 1995), Polish breeds (Zabek et al., 2005), Austrian breeds (Druml et al., 2007), American breeds (Tryon et al., 2009), and Algerian breeds (Berber et al., 2014).

The objective of the study was to characterize the molecular structure, microsatellite polymorphism, and genetic distances among breeds and to draw the phylogenetic tree for horse breeds in Tunisia.

2 Materials and methods

2.1 Population samples

A total of 200 blood samples were taken and tested from the four Tunisian horse breeds (Barb: BA; Arab-Barb: AB; Arabian: AR; and English Thoroughbred: PS). To ensure that each sample was representative of the respective population, a strict sampling strategy was employed and studbook was consulted. The origins and familial relationships of individual animals were considered and 50 samples were taken for each breed.

Analyzed animals were chosen according genealogical information and familiar relationship. Only individuals with different ancestral ascendance were sampled. In order to choose most genetic variability contributors, for each breed, male and female were randomly equally represented.

Genomic DNA was amplified using 16 microsatellite loci (Table 1). All analyzed individuals were registered in the Tunisian breed's studbook. Approximately 5 mL of veins blood per animal was collected aseptically in tubes containing ethylenediaminetetraacetic acid (EDTA, 0.5 mM, pH 8.0).

2.2 DNA isolation and microsatellites selection

Genomic DNA was extracted from total blood using Purelink™ Genomic DNA Mini Kit (Invitrogen) following the manufacturer's protocol. A total of 17 microsatellite markers (Table 1) specific to *Equus caballus* were used in this study. All microsatellite markers are included in the panel recommended by the International Society for Animal Genetics for diversity studies and parentage verification. Alphabetical nomenclature was used for allele size designation in accordance with the International Society for Animal Genetics.

2.3 PCR conditions and data recording

Amplification of used microsatellites included an initial denaturation at 95 °C for 15 min, followed by 30 cycles of 30 s at 94 °C, 90 s at 58 °C annealing temperature, and 1 min at 72 °C. A final elongation step was carried out at 60 °C for 30 min. The amplified products were denatured with formamide (8.3 µL), and Gene Scan-500LIZ (0.3 µL) and PCR products (2 µL) were detected by capillary electrophoresis using an ABI Prism 3130 DNA genetic analyzer (Applied Biosystems, USA). Size analyses of DNA fragments separated were performed with Gene Mapper software (Applied Biosystems, Ver. 4.0). The genotyping assays of microsatellites were performed in the laboratory of Animal Genetic Analysis of the National Institute of veterinary research in Tunis, Tunisia.

2.4 Statistical analysis

Collected molecular data were edited for possible genotyping errors due to null alleles, short allele dominance, typographic errors, and the scoring of stutter peaks. Genetic diversity within breeds, genetic variation, and relationships among breeds were assessed using different softwares. GenAlex 6.2 was used to calculate gene diversity indices for each breed population (Khanshour et al., 2013). Genetix software (version 4.04) was used to screen allelic frequencies and the number of alleles per locus. Observed heterozygosity (H_o), expected heterozygosity (H_e), and unbiased expected heterozygosity (UHe) were calculated across loci and populations (Berber et al., 2014) and the effective number of migrants per generation (N_m ; Wright, 1969). Wright's F statistics (F_{ST} , F_{IS} and F_{IT} ; Wright, 1965, 1978) in the form proposed by Weir and Cockerham (1984) were computed using the Genetix software. The different F statistics look at different levels of population structure. F_{IT} , F_{IS} , and F_{ST} are the inbreeding coefficient of an individual relative to the total population, the inbreeding coefficient of an individual relative to the subpopulation, and the effect of subpopulations compared to the total population, respectively. All coefficients conform to the following equation:

Table 1. Microsatellite sequences and length size.

Loci	Microsatellite sequences	Length size (bp)	References
AHT4	5': AACCGCCTGAGCAAGGAAGT 3': CCCAGAGAGTTTACCCT	144–164	Binns et al. (1995)
AHT5	5': ACGGACACATCCCTGCCTGC 3': GCAGGCTAAGGAGGCTCAGC	126–144	Binns et al. (1995)
ASB2	5': CCACTAAGTGTCGTTTCAGAAGG 3': CACAACCTGAGTTCTCTGATAGG	216–250	Breen et al. (1997)
ASB17	5': ACCATTTCAGGATCTCCACCG 3': GAGGGCGGTACCTTTGTACC	87–129	Breen et al. (1997)
ASB23	5': GAGGGCAGCAGGTTGGGAAGG 3': ACATCCTGGTCAAATCACAGTCC	175–211	Lear et al. (1999)
CA425	5': AGCTGCCTCGTTAATTCA 3': CTCATGTCCGCTTGTCTC	226–246	Eggleston-Stott et al. (1997)
HMS1	5': CATCACTCTTCATGTCTGCTTGG 3': TTGACATAAATGCTTATCCTATGGC	170–186	Guérin et al. (1994)
HMS2	5': CTTGCAGTCGAATGTGTATTAATG 3': ACGGTGGCAACTGCCAAGGAAG	222–248	Guérin et al. (1994)
HMS3	5': CCATCCTCACTTTTTCACTTTGTT 3': CCAACTCTTTGTACATAACAAGA	148–170	Guérin et al. (1994)
HMS6	5': GAAGCTGCCAGTATCAACCATTG 3': CTCCATCTTGTGAAGTGTAACCTCA	151–169	Guérin et al. (1994)
HMS7	5': TGTTGTTGAAACATACCTTGACTGT 3': CAGGAAACTCATGTTGATACCATC	165–185	Guérin et al. (1994)
HTG4	5': CTATCTCAGTCTTGATTGCAGGAC 3': CTCCCTCCCTCCCTCTGTTCTC	127–139	Ellegren et al. (1992)
HTG6	5': GTTCACTGAATGTCAAATTCTGCT 3': CCTGCTTGGAGGCTGTGATAAGAT	84–102	Ellegren et al. (1992)
HTG7	5': CCTGAAGCAGAACATCCCTCCTTG 3': ATAAAGTGCTGGGCAGAGCTGCT	118–128	Marklund et al. (1994)
HTG10	5': TTTTTATTCTGATCTGTACATTT 3': CAATTCCC GCCC ACCCCCGGCA	95–115	Marklund et al. (1994)
VHL20	5': CAAGTCCTTACTTGAAGACTAG 3': AACTCAGGGAGAATCTTCCTCAG	87–105	Van Haringen et al. (1994)

$$(1 - F_{IS})(1 - F_{ST}) = (1 - F_{IT}).$$

The representation of the genetic relationships among tested populations was done using the principal component (PCA) and the factorial correspondence (FCA) analyses as implemented by GenAlex 6.2 and Genetix 4.04. The dendrogram was constructed using unweighted pair group method averages (UPGMA) based on Nei's (1972) genetic distances.

Structure software (Pritchard et al., 2000) was used to analyze the genetic structure of sampled animal. The best k value

corresponding with the number of subpopulation was estimated based on the method of Evanno et al. (2005).

3 Results and discussion

3.1 Microsatellite markers

All loci microsatellites used in this study were amplified successfully in analyzed individuals for the four breeds (Arab-Barb, Barb, Arabian, and English Thoroughbred). Collected data showed no evidence for null alleles or scoring error con-

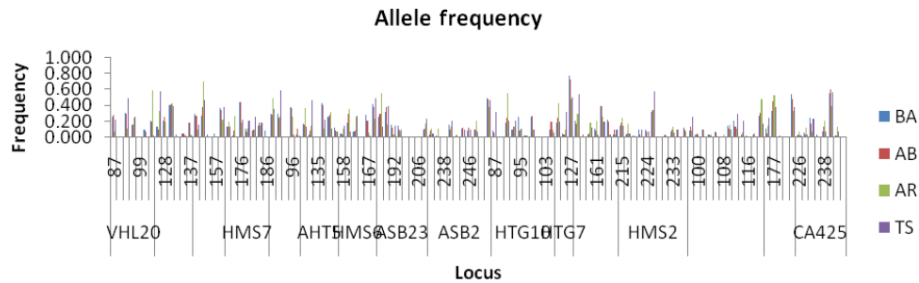


Figure 1. Allelic frequencies by population with graph over loci.

sidering all screened samples, although there were differences among tested samples with regard to presence or absence of alleles and their frequencies. All tested microsatellites were polymorphic in all populations. A total number of 147 different alleles were found across the 16 amplified loci. Allele frequency for analyzed data varied from 0.00 to 0.77. The highest allelic frequency was observed for HTG7 (127 bp) with 0.77 and 0.72 for the Barb and Arab-Barb breeds, respectively. Arabian and English Thoroughbred breeds had the highest frequencies for HMS1 (177 bp) and HMS2 (226 bp) with 0.58 and 0.64, respectively (Fig. 1).

The number of effective alleles per locus indicates low or high genetic polymorphism richness of the used markers. The latter number ranged from 3.20 to 3.78. This parameter was 3.78 (0.31), 3.78 (0.30), 3.41 (0.25), and 3.20 (0.22) in the Arab-Barb, Barb, Arabian and English Thoroughbred, respectively. Although values of the number of effective alleles in studied breeds were in comparable ranges, there is additional morphological variability (muzzle, nose, tail, mane, etc.) that can be detected by other parameters such as mean numbers of alleles per locus, observed heterozygosity, expected heterozygosity, and F statistics.

Genetic diversity within and among breeds

The number of alleles per locus (N_a) varied between 6 (HTG6, AHT6 and HMS1) and 13 (ASB23 and ASB17) with a mean of 9.31 (2.40) alleles. In Barb and Arab-Barb breeds, comparable mean values were found for observed heterozygosity, expected heterozygosity, and unbiased expected heterozygosity. These values were 0.7 (0.03), 0.73 (0.03), and 0.73 (0.03), respectively. For Arabian and English Thoroughbred breeds, respective values for H_o were respectively 0.65 (0.04) and 0.64 (0.03), for H_e they were 0.69 (0.02) and 0.68 (0.02), and for U_{H_e} they were 0.68 (0.02) and 0.68 (0.01). In all analyzed breeds we find H_o lower than H_e . When the observed heterozygosity is smaller than expected, it is because the mating has deviated from random mating-related individuals. Results of this study are similar to those reported by Solis et al. (2005), Khanshour et al. (2013) and Berber et al. (2014). Furthermore, Canon et al. (2000), Tozaki et al. (2003), Behl et al. (2007) and

Kusza et al. (2013) reported 6, 5.8, 5.2 and 6.6 as mean numbers of alleles per locus. However, Haddad et al. (2014) obtained 4.23 as a mean value in Barb and Tunisian Mogod Pony horse breeds.

The mean of the coefficient of inbreeding (F_{ST}) (Wright, 1969) was around 4.9 (0.02) for all analyzed breeds. This result indicates a significant genetic variation between Tunisian horse breeds. Genetic differentiation among breeds was significant ($p < 0.03$) for all loci. The obtained results are comparable to those reported by Berber et al. (2014) and Haddad (2015). The latter authors confirmed the occurrence of genetic differentiation among North African autochthonous horse breeds. In the present study 32 private alleles were found. The number of rare alleles (N_p) was 10, 7, 7 and 8 for BA, AB, AR and PS breeds, respectively (Table 2). Our results confirm findings by Berber et al. (2014) and Haddad (2015), especially for the North African Barb breed.

Mean F_{IS} varied from -0.207 to 0.469 for AR, -0.205 to 0.565 for PS, -0.156 to 0.197 for AB and -0.115 to 0.112 for BA horses. This index reflects the Hardy–Weinberg disequilibrium especially for the Arabian and English Thoroughbred breeds. An important frequency of analyzed loci (62.5 %) showed positive values for F_{IS} . This result confirms that there was mating between relatives. Khanshour et al. (2013) reported similar disequilibrium results for the Arabian breeds.

The F statistics and estimates of number of effective migrants over all populations are given in Table 3 for the four breeds of the study. The individual F_{IS} varied from -0.078 (AHT5 and HMS1) to 0.142 (HMS3). The F_{IS} index was significantly different from zero ($p < 0.05$) only for the Arabian and English Thoroughbred breeds due to a lack of heterozygosity.

Wright (1978) reported that the F_{ST} indicates a moderate differentiation for values between 0.05 and 0.15, a great differentiation for values between 0.15 and 0.25, and an important genetic differentiation for values above 0.25. Overall, F_{ST} values in this study suggest low genetic differentiation in all breeds. The F_{ST} , in our case, ranged from 2.6 to 8.5 % with an average of 5.5 % (0.4). These levels for the F_{ST} in Tunisian horse breeds are smaller than those previously found in Algerian breeds ($F_{ST} = 8.6$ %, Berber et al., 2014),

Table 2. Allelic frequency and heterozygosity for analyzed Tunisian breeds.

Pop		Na	Ne	Ho	He	UHe	F	Np
AB	Mean	7.529	4.109	0.704	0.726	0.733	0.024	10
	SE	0.493	0.320	0.034	0.028	0.028	0.035	
BA	Mean	7.353	4.113	0.689	0.723	0.730	0.049	7
	SE	0.542	0.355	0.034	0.027	0.028	0.025	
AR	Mean	6.294	3.506	0.646	0.690	0.697	0.056	7
	SE	0.435	0.253	0.036	0.023	0.023	0.048	
PS	Mean	6.000	3.434	0.636	0.684	0.691	0.068	8
	SE	0.383	0.244	0.032	0.022	0.022	0.039	
Total	Mean	6.794	3.791	0.669	0.706	0.713	0.049	8.25
	SE	0.242	0.150	0.017	0.012	0.013	0.019	

AB: Arab-Barb; BA: Barb; AR: Arabian; PS: English Thoroughbred; Np: private alleles.

Table 3. F statistics and estimates of F_{IS} , F_{IT} , F_{ST} and N_m over all populations of the Tunisian horse breeds (AB, BA, AR and PS).

	VHL20	HTG4	AHT4	HMS7	HTG6	AHT5	HMS6	ASB23	ASB2	HTG10	HTG7	HMS3	HMS2	ASB17	HMS1	CA425
F_{IS}	0.059	0.080	0.081	-0.014	0.063	-0.078	-0.031	0.072	0.045	-0.002	-0.078	0.142	0.024	-0.025	0.087	-0.065
F_{IT}	0.134	0.144	0.145	0.031	0.126	0.014	0.029	0.109	0.073	0.060	0.000	0.199	0.063	0.018	0.116	-0.037
F_{ST}	0.080	0.070	0.070	0.044	0.068	0.085	0.058	0.040	0.029	0.062	0.072	0.066	0.040	0.041	0.031	0.026
N_m	2.885	3.335	3.314	5.421	3.434	2.683	4.058	6.026	8.290	3.782	3.201	3.528	6.040	5.775	7.727	9.463

Polish breeds ($F_{ST} = 10\%$, Zabek et al., 2005), Brazilian breeds ($F_{ST} = 11.7\%$; Lippi and Mortari, 2003). However, they are slightly lower than the 6.5% reported by Behl et al. (2007) for five Indian horse breeds.

The numbers of effective migrants (N_m) exchanged per generation in all breeds are presented in Table 3. They show that the N_m values for all analyzed samples varied from 2.68 to 9.46. That number of effective migrants was very important especially for the Barb and Arab-Barb breeds (Table 3).

In total, 147 alleles were tested for the Hardy-Weinberg equilibrium (HWE) for all breeds. Significant ($p < 0.05$) deviations from the HWE were observed for 16 (23.5%) of the 68 combinations. Only Arabian and English Thoroughbred horses exhibited significant deviation from the HWE ($p < 0.04$). The Arabian breed showed the maximum number of loci in disequilibrium (10 loci).

3.2 Principal component analysis and spatial interpolation of the results

The first three axes performed on allelic frequencies explain 63.48% of total inertia. Those first three axes explain 25.45, 23.39, and 14.64%, respectively. The special distribution indicates high similarities between Barb and Arab-Barb breeds. A significant difference was observed between the Arabian and English Thoroughbred breeds (Fig. 2).

3.3 Factorial correspondence analysis for analyzed horse breeds

The factorial correspondence analysis as shown in Fig. 3 clearly differentiates the Barb, Arabian, and English Thoroughbred breeds. The Barb and Arab-Barb breeds were clustered together. The Arabian and the Arab-Barb breeds were also grouped together on another cluster with minor similarity compared to the first cluster. Berber et al. (2014) reported genetic proximity of both Barb and Arab-Barb breeds. These results corroborate those advanced by Ouragh et al. (1994). The neighbor-joining clustering approach and the factorial correspondence analysis were used as efficient tools that give precise information on breed relationships (Figs. 3 and 4). Genetic distances, the factorial correspondence, and principal coordinate analyses showed that the significant amount of genetic variation is within population.

3.4 Structure analyses

Estimated individual proportions of membership in each breed are represented by one cluster color. The Arab-Barb (AB) and Barb (BA) breeds are clustered together. The English Thoroughbred (PS) breed was separated from the rest of populations. There are relative similarities between the Arab-Barb (AB) and the Arabian (AR) breed.

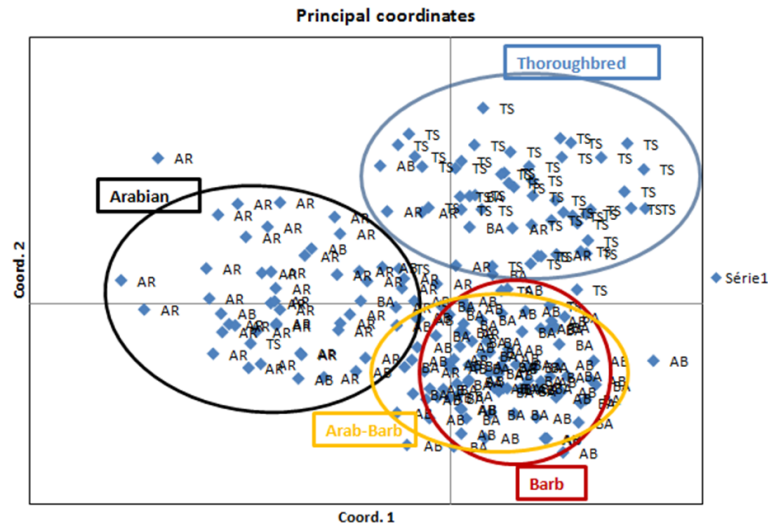


Figure 2. Principal component analysis for the Tunisian horse breeds.

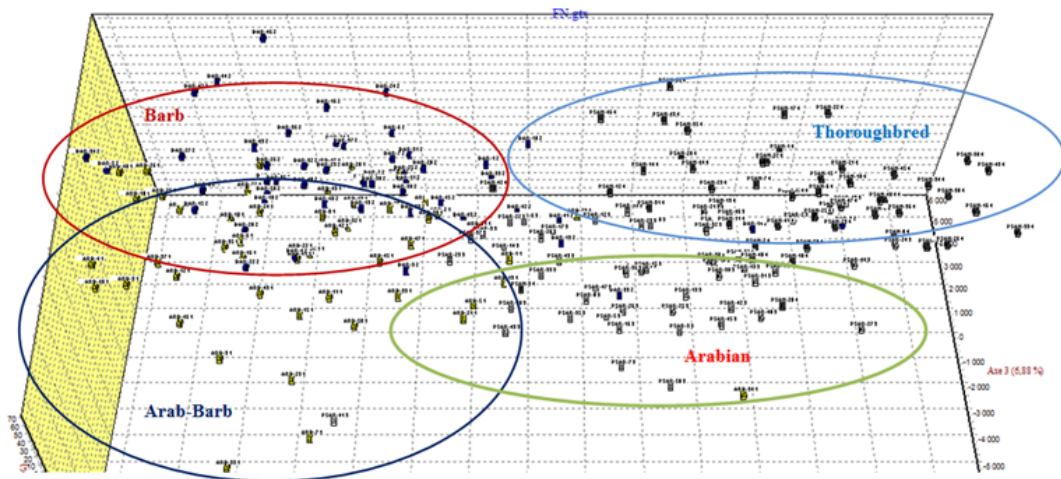


Figure 3. Factorial correspondence analysis for the Tunisian horse breeds.

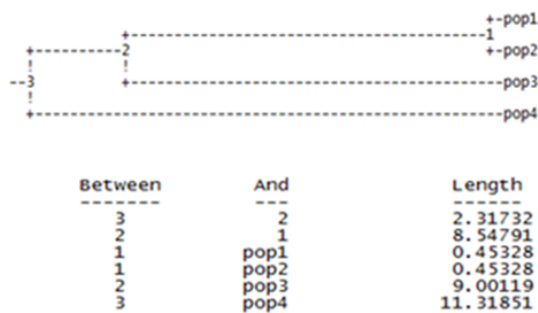


Figure 4. The neighbor-joining dendrogram including the four studied Tunisian breeds. AB: Arab-Barb (pop1); BA: Barb (pop2); AR: Arabian (pop3); and PS: English Thoroughbred (pop4).

4 Conclusion

This paper highlights the genetic structure of the Tunisian horse breeds. There is a genetic differentiation between Tunisian Barb and Arab-Barb horses and other breeds. The Barb and Arab-Barb appeared to be genetically similar and considered as the same group. This is can be explained by the continuous gene flow between both breeds. Furthermore, the significant amount of genetic variation was within populations. These results may help in the implementation of conservation programs of Tunisian horse breeds and enhance efforts to improve preserving revealed genetic diversity.

Data availability. The data used in the study can be obtained by email (Mezir Haddad, mezirhaddad@yahoo.fr) from the Fondation Nationale d'Amélioration de la Race Chevaline Sidi Thabet.

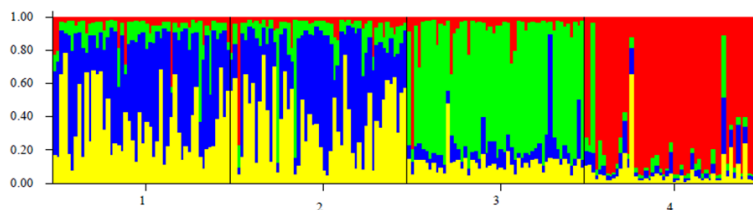


Figure 5. Graphical representation of membership of 200 individuals from AB: Arab-Barb (1); BA: Barb (2); AR: Arabian (3); and PS: English Thoroughbred (4).

Competing interests. The authors declare that they have no conflict of interest.

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