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ARTICLE

Genotypic study of Tunisian Arabian stallions

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ABSTRACT Diversity of Arabian stallions distributed throughout the territory of Tunisia has been evaluated. Seventeen microsatellite markers were used for the determination of genetic variation. A total of 95 alleles were detected in the 50 stallions studied. The number of alleles per locus ranged from 2 to 9 with an average of 5.6. The observed mean number of genetically diverse alleles (Na) was 5.6 (0.44). The mean number of expected alleles (Ne) was 3.42 (0.28). The average expected and observed heterozygosity was approximately 0.675 (0.026) and 0.593 (0.044), respectively. Principal component analysis showed the presence of 2 subpopulations in the studied sample set. These findings demonstrate the potential of microsatellites as a tool for designing and controlling animal breeds. Results show that the population under study has sufficient levels of genetic variations, which can be used as a foundation for developing plans for species conservation and long-term sustainability.
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Introduction

In North African nations' history, culture, and economy, horses play a significant role. There are five distinct breeds of horses found in Tunisia, including the Barb (BA), Arab-Barb (AB), Arabian (AR), Thoroughbred English (PS), and Pony of the Mogods (PM) (Jemmali et al. 2015). The overall number of horses in Tunisia is estimated to be 37 000 by the National Foundation for the Improvement of the Horse Race (FNARC) (FNARC 2015), with only 5000 belonging to the Purebred Arabian breed. The bulk of these horses are popularly known by the designations BA and AB. Complete knowledge of genetic diversity and population structure of the horses is crucial due to the significance of Tunisian horse breeds as it serves as the foundation for all conservation and sustainable management activities (Notter 1999). In general, numerous techniques are used to characterize animal breeds, including phenotypic charaterization (morphobiometric traits), biochemical (protein polymorphism), cytogenetic (number and structure of chromosomes), and molecular (molecular genetic analysis). DNA polymorphisms analysis became a widely used technique to the study of genetic structure and the evolutionary history of animals in recent years. Molecular markers have emerged as novel methods for analyzing genetic diversity. Genetic variability, consanguinity, and gene flow (Buchanan et al. 1994; Moazami-Goudarzy et al. 1997), DNA fingerprinting, and mapping, have all become more widely available and methods for assessing these polymorphisms have multiplied (Bishop et al. 1994). Because of the benefits they provide, particularly in terms of conservation, microsatellites swiftly gained the status of favored markers in population genetics (Cañon et al. 2001; Rognon X and Verrier 2007). Microsatellites, by definition, are DNA sequences that contain tandem repeats of a 1-6 bp motif (Chambers and MacAvoy 2000; Vieira et al. 2016). They are frequently employed in the study of genetic variation among various animal breeds, including camelids, horses, and cattle (Berber et al. 2014), cows (Baccouche 2014), and ovines (Ciani 2013; Nouairia 2015). These sequences are quite numerous and evenly dispersed across the genome, and they are distinguished by substantial polymorphism since the amount of repeats varies depending on the alleles (Boichard et al. 1998). Microsatellites are the perfect markers study of genetic diversity due to their nearly uniform distribution throughout the genome. Numerous research studies have demonstrated the value of microsatellites for assessing genetic diversity and conducting livestock breed conservation studies (Boichard et al. 1998; Mburu et al. 2003; Buchanan et al. 1994; MacHugh et al. 1998; Saitbekova et al. 1999; Hanotte et al. 2000; Hanslik et

al. 2000; Mburu et al. 2003).

This research deployed microsatellite markers to examine the genetic diversity of the purebred Arabian horse population in several Tunisian regions.

Materials and methods

Samples and DNA extraction

A total of 50 purebred Arabian stallions were part of this work. Blood samples of stallions were collected from the jugular vein in 5 ml tubes containing EDTA (1.8 mg/ml). DNA extraction was done with the Purelink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA).

Microsatellites used

A set of 17 microsatellite markers was chosen for the genotyping of the Tunisian Arabian breed to characterize intrapopulation genetic variability (Table 1). These markers are part of the list recommended by the ISAG-FAO Advisory Group for the analysis of genetic diversity, identification and parentage control of equine breeds, on a global scale (FAO 2011).

DNA amplification by PCR

Genomic DNA was amplified by multiplex PCR using the Stocks Marks for Horses, Equine Genotyping Kit (Applied Biosystems, Foster City, California, USA) using 17 microsatellites loci (Table 1) according to FAO guidelines (2011). The optimized PCR conditions were the followings: 10 min of initial denaturation at 95 °C, followed by 30 cycles of 30 s at 95 °C, with the annealing temperature at 60 °C for 30 s, and the extension for 1 min at 72 °C. A final extension step was carried out at 72 °C for 1 hour to amplify the used microsatellites. The reaction volume used for each 25 µl sample contained 2.5 µl $MgCl_2$ (25 mM), 0.2 µl dNTP (25 mM), 0.5 µl of each primer (25 pmol/µl), 0.2 µl of Taq polymerase (5 U/µl) , 50 ng DNA and 2.5 µl of buffer (5X). Amplified products were denatured with HiDi TM formamide (8.3 µl). PCR products (2 µl) were separated by capillary electrophoresis using an ABI Prism 3130 DNA genetic analyzer (Applied Biosystems, USA), and the size analysis of DNA fragments was performed using Gene Scan-500 LIZ (0.3 µl) and the Gene Mapper Software (Applied Biosystems, Ver. 4.0).

Parameters of genetic diversity

The level of intrapopulation variability was used to examine the genetic variety within the studied population. The Hardy Weinberg's law was applied to determine whether a population was in equilibrium. We calculated seven parameters to characterize the genetic diversity. Direct comparison of allele frequencies is challenging when considering the genetic heterogeneity within populations. Utilizing the GenAlex program (version 6.2), the following were calculated:

- Polymorphism rate (P)
- Number of alleles per locus (A)
- The observed and expected number of alleles for microsatellites (Na and Ne)
- Observed and expected heterozygosity (Ho and He)
- Fixation index (Fis)

Polymorphism rate (P): This parameter is the percentage of polymorphic loci in the sample studied.

The probability of detecting at least two alleles at a specific locus is determined by the relative frequency of the allele as well as the sample size. A locus is considered polymorphic in the present study if the most frequent allele has a frequency of less than or equal to 0.95.

The number of alleles per locus (A): This parameter represents the number of allele in the tested population, it is calculated according to the formula:

$$A = 1/L \Sigma a$$

a: represents the number of alleles at a locus
L: represents the number of loci studied.

Observed heterozygosity (Ho): is the proportion of heterozygous individuals at locus K as in the formula:

$$Hok=\Sigma_{i,j=1} Pij(i \neq j)$$

Pij: is the estimate of the frequency of genotype ij at locus k and
ak: the number of alleles at locus k.

If we consider the L locus, the rate of observed heterozygosity (Ho) is the average of (Hok) according to the equation:

$$Ho=1/L\Sigma_{k=1}^{L} Hok$$

Expected heterozygosity (He): can be calculated from allelic frequencies determined for each locus using the formula:

$$He = 1-\Sigma p^2 i$$

Pi is the frequency of allele L at this locus.

The average rate of heterozygosity is the most characteristic phenomenon of genetic diversity. Its numerical value depends on the number of polymorphic loci and the genetic diversity of each of them.

Table 1. Microsatellites used for the characterization of the intrapopulation.

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'CACAACTGAGTTCTCTGATAGG	216-250	Breen et al. (1997)
ASB17	5'ACCATTCAGGATCTCCACCG 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'CTTGCAGTCGAATGTGTATTAAATG 3'ACGGTGGCAACTGCCAAGGAAG	222-248	Guérin et al. (1994)
HMS3	5'CCATCCTCACTTTTTCACTTTGTT 3'CCAACTCTTTGTCACATAACAAGA	148-170	Guérin et al. (1994)
HMS6	5'GAAGCTGCCAGTATTCAACCATTGG 3'CTCCATCTTGTGAAGTGTAACTCA	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'ATAAAGTGTCTGGGCAGAGCTGCT	118-128	Marklund et al. (1994)
HTG10	5'TTTTTATTCTGATCTGTCCACATTT 3'CAATTCCCGCCCCACCCCCGGCA	95-115	Marklund et al. (1994)
VHL20	5'CAAGTCCTCTTACTTGAAGACTAG 3'AACTCAGGGAGAATCTTCCTCAG	87-105	Van Hearingen et al. (1994)

Fixation index (Fis): This parameter measures the difference between observed heterozygosity (Ho) and expected heterozygosity (He) and is calculated as it is mentioned in the formula:

$$Fis=1-Ho/He$$

The genotype frequency of the tested population is known as the Hardy-Weinberg equilibrium because it occurs at random with regard to the genotypes of these gametes at the given locus in a population of individual's diploids. The fixation index, which ranges from -1 to +1, measures the deviation from the Hardy-Weinberg structure and reveals the heterozygotes-deficit for each population, each locus, and all the loci. When there is an imbalance between the panmictic and heterozygote fractions in the population, F is positive; when there is an imbalance, it is negative. The difference is the result of a variety of processes, including inbreeding, drift, selection, differentiation, etc.

Finally, principal component analysis (PCA) was performed via plotting these data in two dimensions to visualize clusters of related data points.

Results and discussions

Polymorphism rate

A locus is polymorphic at the threshold of 5% if it has at least two different alleles. In our study, the polymorphism rate is 100% at the threshold of 5%, so all the loci examined were polymorphic. Each locus tested is represented by

Table 2. The observed number of genetically diverse alleles (Na) and expected number of alleles (Ne) for each microsatellite used under the hypothesis of Hardy-Weinberg equilibrium.

	VHL20	HTG4	AHT4	HMS7	HTG6	AHT5	HMS6	ASB23	ASB2	HTG10	HTG7	HMS3	HMS2	ASB17	LEX3	HMS1	CA425
N	50	50	50	46	48	50	47	24	42	50	46	50	40	23	16	21	17
Na	5	4	3	8	4	5	6	7	9	6	4	7	7	6	7	2	5
Ne	2,841	2,606	2,065	5,496	3,252	3,043	3,865	3,692	5,018	3,828	2,333	4,244	3,540	2,875	5,505	1,995	1,920

Table 3. The expected (He) and observed (Ho) heterozygosity for each microsatellite used under the hypothesis of Hardy-Weinberg equilibrium

	VHL20	HTG4	AHT4	HMS7	HTG6	AHT5	HMS6	ASB23	ASB2	HTG10	HTG7	HMS3	HMS2	ASB17	LEX3	HMS1	CA425
Ho	0,700	0,600	0,480	0,717	0,604	0,700	0,660	0,583	0,738	0,760	0,674	0,660	0,675	0,696	0,063	0,476	0,294
He	0,648	0,616	0,516	0,818	0,692	0,671	0,741	0,729	0,801	0,739	0,571	0,764	0,718	0,652	0,818	0,499	0,479

more than one allele, which promotes genetic diversity. This indicates the effectiveness of using microsatellite markers for the determination of the parameters of the genetic diversity of the examined stallion samples.

Number of alleles per locus: allelic richness (A)

Allelic diversity of a population is defined as the number of alleles present in a locus. It is known to depend on the sample size, since the chances of discovering a new allele increase each time a new individual is tested (Foulley et al. 2006). In this study, the total number of alleles observed for the 17 tested microsatellites is 95 and the average number of alleles per locus is 6 (5.58).

The observed and expected number of alleles for microsatellites (Na and Ne)

The mean number of observed alleles (Na) is 5.6 (0.44). The mean number of expected alleles (Ne) is 3.42 (0.28). The number of observed alleles (Na) for microsatellites analysis varied from 2 for the HMS1microsatellite to 9 for the ASB2 microsatellite (Table 2). The highest expected number of alleles (Ne) is 5.5 for the LEX 3 microsatellite and the lowest is 1,920 for the CA425microsatellite (Table 2). According to these results, the number of observed alleles (Na) is higher than expected (Ne) for all the amplified loci so the population is heterogeneous which confirms an intrapopulation genetic diversity.

Heterozygosity rate (Ho, He)

The observed and expected heterozygosity rates were calculated for each locus under the hypothesis of Hardy-Weinberg equilibrium. To estimate the level of genetic polymorphism, we compared the two rates of heterozygosity. The observed heterozygosity (Ho) for analyzed microsatellites varies between 0.063 for LEX 3 to 0.760 for HTG10 with an average of 0.593 ± 0.044 alleles. It is higher than that found in other horse breeds (H0 = 0.026 ± 0.07) (Hadded et al. 2014) (Table 3). There is a large variability of Ho. This confirms that investigated loci contain different numbers of alleles. The expected heterozygosity (He) for the analyzed microsatellites varies between 0.479 for CA425 to 0.818 for LEX with an average of 0.675 ± 0.026, which is higher than the reported ones in other horse breeds (He = 0,326 ± 0,026) (Hadded et al. 2014). This fluctuation shows the variability of the number of alleles in each locus. We contrasted the two rates of heterozygosity in order to evaluate the importance of genetic polymorphism. The expected heterozygosity for the population based on many loci is higher than what was observed, reflecting a positive difference that may suggest a deficit of heterozygosity in the population.

Fixation Index (Fis)

This parameter is the measure of the difference between the population of individuals found in the heterozygous (HO) state and the expected heterozygous (HE) in a population found away from the Hardy-Weinberg equilibrium. If populations studied are in the equilibrium of HW, then Fis = 0. If Fis is negative, the populations present an excess of heterozygosity. The observed Fis in individuals (analyzed

Table 4. Fixation index observed with microsatellites showed an excess in heterozygotes for five loci (Fis < 0)) and deficit in heterozygotes (Fis > 0) for the other loci

	VHL20	HTG4	AHT4	HMS7	HTG6	AHT5	HMS6	ASB23	ASB2	HTG10	HTG7	HMS3	HMS2	ASB17	LEX3	HMS1	CA425
FIS	**-0,080**	0,026	0,069	0,123	0,128	**-0,043**	0,110	0,200	0,078	**-0,029**	**-0,179**	0,137	0,059	**-0,067**	0,924	0,045	0,386

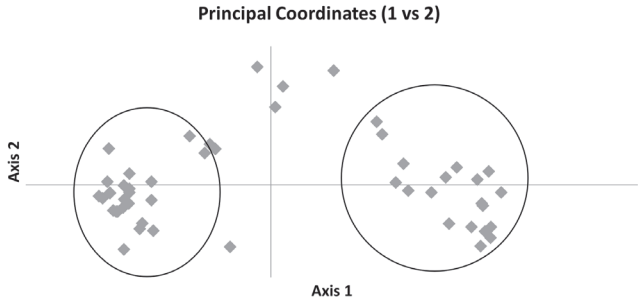

Figure 1. Result of the CPA of the studied stallions. AR: Arabian stallion. Axis 1 (38%) Axis 2 (18%).

with microsatellites) varies between - 0.179 for HTH7 (excess in heterozygotes) and 0.924 for LEX3 (deficiency in heterozygotes) (Table 4). Fis is different from 0 and they are negative for some analyzed markers which are VHL20, AHT5, HTG10, HTG7, and ASB17. These results indicate an excess of heterozygosity. The average fixation index in our study is in order 0.11 (0.059) and this low value also confirms the importance of genetic diversity in the tested stallions.

Principal component analysis

Principal component analysis confirmed the observed genetic diversity. For the 50 stallions, we noticed the existence of 2 subpopulations. Axis 1 and Axis 2 explained 38% and 18% of the variation in a population, respectively. These results show that there is no significant genetic diversity which can be explained by the absence of randomized crossbreading.

Conclusion

These presented results constitute a contribution to the characterization of purebred Arabian horses in Tunisia. This work could thus serve as a basis for more extended studies to describe the genetic diversity of their genetic diversity. Several factors including the practices of breeders, the current status of the species and their evolutionary history explain its current genetic diversity. Describing the existing genetic variation within the population will be an important information for further breeding activities.

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