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Genetic Characterization and Bottleneck Demographic Assessment of Caspian Horse Population

KARAKTERISASI GENETIK DAN HAMBATAN DEMOGRAFI PADA PENILAIAN POPULASI KUDA KASPIA

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ABSTRAK

Tujuan dari penelitian ini adalah untuk mengevaluasi karakter genetik populasi kuda kaspia menggunakan penanda mikrosatelit. Penelitian ini berkeinginan menentukan efisiensi penanda mikrosatelit untuk suatu rencana konservasi dan strategi pemuliaan dalam populasi kuda kaspia. Sebanyak 120 sampel kuda kaspia, terdiri dari 95 kuda dewasa dan 25 kuda anakan diberi genotip dengan menggunakan 17 penanda mikrosatelit yang direkomendasikan oleh ISAG. Jumlah alel per lokus bervariasi dari lima (HMS01 dan HTG07) hingga sembilan (HTG10) dengan rataan 7,41. Heterozigositas yang diamati berkisar 0,505-0,831 (rataannya 0,684), dan heterozigositas yang diharapkan berkisar 0,615-0,835 (rataannya 0,748). Nilai PIC berkisar antara 0,716 (HMS01) hingga 0,834 (AHT04) dengan rataannya 0,787. Probabilitas eksklusi total 17 lokus mikrosatelit adalah 0,9999. Nilai rendah dari Indeks fiksasi Wright / Fis (0,084) menunjukkan bahwa berlangsung tingkat *inbreeding* yang rendah. Suatu keadaan heterozigot yang secara nyata berlebihan berdasarkan model yang berbeda, menunjukkan bahwa populasi kuda kaspia telah menurun ke angka rendah di masa lalu, tetapi kejadian *bottleneck* masih sangat mencolok, dan jumlahnya telah meningkat namun tidak dalam *mutation drift equilibrium*. Penelitian ini berkontribusi pada pengetahuan kita tentang keragaman genetik pada populasi kuda kaspia dan membantu untuk menentukan strategi konservasi genetiknya.

Kata-kata kunci: karakterisasi genetik; penanda mikrosatelit; kuda kaspia

ABSTRACT

The aim of this study was to evaluate genetic characterization of the Caspian horse population using microsatellite markers. This study was determined the efficiency of microsatellite markers for conservation plans and breeding strategies in Caspian horse population. A total of 120 Caspian horse samples including 95 adults and 25 foals were genotyped by using seventeen microsatellite markers recommended by ISAG. The number of allele per locus varied from 5 (*HMS01* and *HTG07*) to 9 (*HTG10*) with an average of 7.41. The observed heterozygosity and the expected heterozygosity ranged from 0.505-0.831 (mean 0.684), from 0.615-0.835 (mean 0.748) respectively. PIC value ranged from 0.716 (*HMS01*) to 0.834 (*AHT04*) with an average of 0.787. The total exclusion probability of 17 microsatellite loci was 0.9999. The low values of Wright's fixation index/ F_{is} (0.084) indicated the low levels of inbreeding. A significant heterozygote excesses based on different models, suggested that Caspian horse population has decreased to low numbers in the past, but a bottleneck event is still very striking, and its number has recently increased is not in mutation drift equilibrium. The present study contributes to our knowledge of the genetic diversity of the Caspian caspian horse population and helps to deûne its genetic conservation strategies.

Keywords: genetic characterization; microsatellite markers; Caspian horse

INTRODUCTION

There are more horse breeds in the world than in the era before mechanization, when horses become the driving force behind all transport and agriculture. Managing and tracking of its individuals requires a system of identification that is not interested and is internationally changeable. The FAO reported that many horse were suffered from decreasing in their number. It is important to evaluate the genetic variability in horse population in order to develop conservation programs (Mahrous *et al*., 2011). Iran has a long history of horse domestication and breeding (Rafeie *et al*., 2011). Iranian horses were classified into 4 groups according to their origins: Northern alluvial plain such as Caspian horses, northeast areas such as Turkmen breed, and western highlands such as Kurd breed and southwestern and central areas such as Persian-Arab breed (Rafeie *et al*., 2011). The Caspian horse is a beautiful creature with a wonderful temperament. They have a beautiful rhythm which, making them become a favorable ponies show (Seyedabadi *et al*., 2006). This breed is quite small, typically ranging from 9-10 height handed. The Caspian horse was rediscovered in 1965. Today the estimated number of Caspian horses is approximately 250 individuals (Seyedabadi *et al*., 2006). Studbook data includes some errors in the registration of the Caspian studbook. Those data important to the conservation of Caspian horses and true ancestral lineage might be essential for breeding of this breed. Another difficulty in horse breeding program is loss of genetic variation and increases in inbreeding. The effects of inbreeding in population cause a decrease in genetic variation and limiting the potential genetic benefits of artificial selection. Genetic analysis using molecular markers can provide valuable information about current levels of genetic variation. This information can be used to estimate which specific management strategies will influence genetic variation in population. Microsatellite markers are a class of genetic markers commonly used for population studies and parentage verification. Microsatellite markers were ûrst characterized in Swedish horse (Ellegren *et al*., 1992; Marklund *et al*., 1994). Due to their high level of polymorphism and co-dominant inheritance, microsatellites are used in individual identification and parentage control. DNA markers could be used for examination of genetic structure of populations, estimation of degree of inbreeding, homozygosity, genetic distance between populations, planning of crossbreeding programs and conservation programs (Fornal *et al*., 2013). Generally, a seventeen set of microsatellites loci are used in horses. Those markers is a locus panel were recommended by International Society for Animal Genetics (ISAG) in horses parentage testing. The polymorphism of these markers has been proved to be useful in Iranian horse breeds such as, Turkmen horse (Rahimi Mianjia *et al*., 2015); Iranian-Arab horse (Moshkelani *et al*., 2011) and Kurd horse (Rafeie *et al*., 2011). Application of microsatellite markers in evaluation of the genetic structure in Caspian horses has not been done yet and this is the first research for parentage verification based on seventeen microsatellites loci recommended by ISAG's in this breed. The purpose of this study was to obtain genetic information on the genetic variability to identify bottleneck events of endangered Caspian horses using analysis of 17 microsatellite loci and design a marker system for future low-cost genotyping, which will give high combined exclusion probabilities (EPs).

RESEARC METHODS

Sample

The sample were chosen by their breeders which has a pedigree document (parents, offspring). Blood samples were collected from 120 Caspian horses consist of 95 adults (36 stallions and 59 males) and 25 foals. Genomic DNA was extracted from blood samples using the salting-out method (Miller *et al*., 1988).

Microsatellite markers genotyping

A panel of 17 microsatellite markers was selected for this study that had been recommended by ISAG for individual identification and parentage verification of Caspian horses (Table 1).

The 17 microsatellites were amplified in 2 multiplex reactions. Each reaction had a final volume of 20 µL, containing 40 ng of genomic DNA, 2 mM $MgCl_2$ (Fermentas, Canada), 250 µM of each dNTP (Roche Applied Science, Germany), 0.03 µM of both primers (Metabion, Germany), 1X PCR buffer (Fermentas, Canada) and 0.5U Taq DNA polymerase (Fermentas, Canada). Amplifications were performed using the Eppendorf Mastercycler 1659. PCR amplification was as follows: the first step was

Table 1. Characteristics of 17 Horse microsatellites DNA loci

Loci	Primer sequences 5'-3'	Dye	Size Range(bp)
AHT04	F: AACCGCCTGAGCAAGGAAGT R: CCCAGAGAGTTTACCCT	6-Fam	166 - 140
AHT05	F: ACGGACACATCCCTGCCTGC R: GCAGGCTAAGGGGGCTCAGC	VIC®	147 - 126
ASB02	F: CCTTCCGTAGTTTAAGCTTCTG R:CACAACTGAGTTCTCTGATAGG	VIC®	268 - 237
ASB17	F: GAGGGCGGTACCTTTGTACC R: ACCAGTCAGGATCTCCACCG	PET®	116 - 104
ASB23	F: GAGGTTTGTAATTGGAATG R: GAGAAGTCATTTTTAACACCT	VIC®	212 - 176
HMS01	F: CATCACTCTTCATGTCTGCTTGG R:TTGACATAAATGCTTATCCTATGGC	PET®	178 - 166
HMS02	F: ACGGTGGCAACTGCCAAGGAAG R:CTTGCAGTCGAATGTGTATTAAATG	NED™	236 - 215
HMS03	F: CCAACTCTTTGTCACATAACAAGA R: CCATCCTCACTTTTTCACTTTGTT	NED™	170 - 146
HMS06	F: GAAGCTGCCAGTATTCAACCATTG R: CTCCATCTTGTGAAGTGTAACTCA	VIC®	170 - 154
HMS07	F: CAGGAAACTCATGTTGATACCATC R:TGTTGTTGAAACATACCTTGACTGT	6-FAM™	187 - 167
HTG04	F: CTATCTCAGTCTTCATTGCAGGAC R: CTCCCTCCCTCCCTCTGTTCTC	6-FAM™	137 - 116
HTG06	F: CCTGCTTGGAGGCTGTGATAAGAT R: GTTCACTGAATGTCAAATTCTGCT	VIC®	103 - 74
HTG07	F: CCTGAAGCAGAACATCCCTCCTTG R: ATAAAGTGTCTGGGCAGAGCTGCT	NED™	128 - 114
HTG10	F: CAATTCCCGCCCCACCCCCGGCA R: TTTTTATTCTGATCTGTCACATTT	NED™	110 - 83
LEX33	F: TTTAATCAAAGGATTCAGTTG R: TTTCTCTTCAGGTGTCCTC	PET®	217 - 203
UCDEQ425	F: AGCTGCCTCGTTAATTCA R: CTCATGTCCGCTTGTCTC	PET®	247 - 224
VHL20	F: CAAGTCCTCTTACTTGAAGACTAG R: AACTCAGGGAGAATCTTCCTCAG	6-FAM™	102 - 83

performed by initial denaturation for 5 min at 95°C, followed by 35 cycles at 95°C for 30sec, 58°C or 60°C for 30 sec, and 72°C for 1 min then extension step of 72°C. The set of proofreading activity and fluorescently labeled 17 primers specific for STRs was tested. PCR products were further sequenced using capillary electrophoresis system on the 3130×l Genetic Analyser (Applied Biosystems). The GeneScan-500 LIZ Size Standard was used in each sample run for an application of automated DNA fragments analysis with four fluorescent dyes. Analysis of DNA profiles for 17 STR loci was conducted in GeneMapper 4.0 software (Applied Biosystems).

Data analysis

Software CERVUS version 3 (Kalinowski *et al*., 2007) was used to calculate number of alleles (Na), Allele's frequencies for each locus, observed heterozygosity (H_O), expected heterozygosity (He), Polymorphic information content (PIC) and combined probability of exclusion (PE). Deviations from HWE and inbreeding coefficient (F_{is}) were estimated by PopGene version 4.4 program (Rousset, 2008). Bottleneck events was studied in Caspian horse population by estimating the heterozygosity excess using software BOTTLENECK (http: //www.ensam. inra.fr/ URLB). Three tests: Sign, Standardized

differences and Wilcoxon sign-rank tests under three models (IAM, SMM and TPM) were used to compute the distribution of gene diversity expected from the observed number of alleles, given sample size under the assumption of mutation–drift equilibrium (Cornuet and Luikart, 1998).

RESULTS AND DISCUSSION

The overall range data and mean values for observed number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He) and polymorphic information content (PIC), showed a high genetic diversity in the Caspian horses population (Table 2) and all the microsatellite used were polymorphic in this breed.

The number of allele per locus varied from 5 (*HMS01* and *HTG07*) to 9 (*HTG10*) with an average value of 7.41 in the Caspian horse. The observed heterozygosity and the expected heterozygosity ranged from 0.505-0.831 (mean 0.684), from 0.615-0.835 (mean 0.748) respectively. Microsatellite markers showing PIC values higher than 0.7 are commonly considered as informative in horse population (Rukavina *et al*., 2016). All marker in this study were informative since the average PIC value calculated at 0.787. The lowest PIC value was for *HMS01* (0.716), while the highest value was for *AHT04* (0.834). The mean estimated value for Fis was 0.084 and among loci varied from 0.367 (*ASB17*) to –0.177 (*HTG10*). The within population inbreeding estimate (F_{is}) ranged between –0.177 and 0.367 with an average of 0.084. Thus, on an average, deficiency (%8.4) of heterozygote existed in the Caspian horse population (Table 2). Statistically significant deviation from Hardy-Weinberg equilibrium ($P<0.05$) was found at total loci, except for loci *AHT04*, *AHT05*, *ASB02*, *HMS03* and *HTG4* (Table 2). The obtained PE for each polymorphic locus was ranged from 0.426 for *HMS01* to 0.813 for *HTG10* with a combined average probability of exclusion of 0.99999 (Table 2). The parentage testing of the 25 foals was verified by the compatibility of seventeen microsatellite markers according to Mendelian laws and using likelihood based method. However, 4 foals did not inherit alleles from the registered sire and 1 foal did not inherit alleles from the registered dam. Microsatellite data were used for statistical analysis to determine whether the population had undergone genetic bottlenecking in recent times (100–200 generations). The expected number of loci with heterozygosity excess was 9.88, 9.55,

Table 2. Number of alleles (Na), observed heterozygosity (H_o), expected heterozygosity (H_e), Polymorphic information content (PIC), inbreeding coefficient (F_{is}), exclusion probabilities (PE) and Hardy Weinberg Equilibrium (HWE) of 17 microsatellites loci for Caspian horse.

Loci	Na	H_O	H_e	PIC	F_{is}	PE	HWE
AHT04	8	0.831	0.805	0.834	-0.032	0.685	NS
AHT05	8	0.712	0.715	0.818	0.004	0.625	NS
ASB02	8	0.762	0.723	0.752	-0.054	0.645	NS
ASB17	8	0.505	0.798	0.785	0.367	0.633	**
ASB23	7	0.625	0.705	0.724	0.113	0.576	**
HMS01	5	0.512	0.721	0.716	0.290	0.426	**
HMS02	8	0.712	0.753	0.827	0.054	0.636	*
HMS03	8	0.824	0.815	0.801	-0.011	0.623	NS
HMS06	7	0.723	0.781	0.791	0.075	0.553	*
HMS07	8	0.575	0.835	0.785	0.311	0.633	**
HTG04	8	0.732	0.715	0.758	-0.023	0.620	NS
HTG06	7	0.817	0.732	0.804	-0.116	0.531	**
HTG07	5	0.514	0.711	0.738	0.277	0.429	**
HTG10	9	0.745	0.615	0.824	-0.177	0.813	**
LEX33	8	0.682	0.801	0.798	0.148	0.721	**
UCDEQ425	6	0.612	0.703	0.799	0.130	0.615	**
VHL20	8	0.754	0.812	0.827	0.071	0.683	*
Mean ± SD	7.41±0.23	0.684±0.05	0.748±0.15	0.787±0.11	0.084±0.04	0.9999	-

and 8.57 for IAM, TPM, and SMM, respectively. The number of loci with observed heterozygosity excess under this test was 15, 13, and 3 for the above-mentioned models. The null hypothesis was rejected when using the Sign test and indicated a recent genetic bottleneck. The second method used was the standardized difference test. The hypothesis of mutation-drift equilibrium was rejected in the IAM (P=0.002) model; however, it was accepted in the TPM model (P=0.255). The Wilcoxon test, which gives high statistical power and can be used for four polymorphic loci and any number of individuals, also indicated an excess in heterozygosity and bottlenecks in two models with probability values of 0.001 (IAM) and 0.048 (TPM). However, the null hypothesis was accepted under SMM (0.989) (Table 3).

Most historical arguments for animal conservation do not depend on genetic information, but microsatellite markers can reveal the levels of past breeding. As there are only a few genetic studies on Caspian horse population, the aims of this study was to supply new information to equine breeder about the population structure, genetic characterization, and genetic background of the Caspian horse. In total, 126 alleles were detected on the loci, with a mean of 7.411 alleles per locus. This mean number of alleles per locus was higher than that reported in the Iranian Arabian horse (4.29), Arabian horse breed from Syria (5.69), Italian horses (7.01) and Spanish Trotter horses (6.0) (Moshkelani et al., 2011; Khanshour and Cothran, 2012; Azor et al., 2007) but lower than reported in the Brazilian Criollo Horse (13.6 ± 0.6), Iranian Turkmen Horse (9.42) and Arabian horses in Egypt (7.563) (Rahimi Mianjia *et al*., 2015; Costa *et al*., 2010; Georgescu *et al*., 2005). The reasons for the different mean number of alleles might be variation in the number of microsatellites, number of sample, or the population structure of the different breeds. Total microsatellite markers were applied in this study showed reliable polymorphism for evaluating genetic variation within the Caspian horse population. The results showed that the Caspian horse population has a moderate level of heterozygosity (0.684). Compared with other mean observed heterozygosity values, this value was higher than in Egyptian Arabian horse (0.631), Arabian horse from Bosnia and Herzegovina (0.629) and Thoroughbred (0.681) but lower than in Hucal horse (0.702), Korean native horse (0.703), and Iranian Turkmen Horse (0.757) (Fornal *et al*., 2013; Rahimi Mianjia *et al*., 2015; Georgescu *et al*., 2005; Rukavina *et al*., 2016; Cho, 2006). In this study the mean value of observed heterozygosity was lower than the mean of expected heterozygosity that is 0.684 and 0.748, respectively, indicating that the studied population represents a narrow genetic base of the Caspian horse breed. When a population is exposed to a bottleneck, the observed heterozygosity would be larger or smaller than the expected heterozygosity (Cornuet and Luikart, 1998). Fornal *et al.* (2013) showed that seventeen loci had higher H_o than H_e values in the Hucul horse population. Five (*AHT04*, *AHT05*, *ASB02*, *HMS03* and *HTG04*) of the 17 loci did not show signiûcant deviations from HWE due to an excess of heterozygotes. The Polymorphism Information Content (PIC) similar to heterozygosity and is calculated from allele frequencies. A high PIC value is indicative of a locus with high informativeness. In this study average PIC value was 0.787 which is high polymorphic. Dierks *et al*. (2007), selected microsatellite markers with PIC values > 0.5 as markers with values below this level are insufficient for parentage verification. The inbreeding index (Fis) indicates moderate level of inbreeding in Caspian horse population, but F_{is} for locus *HMS01*, *HMS07*, *HTG07* and *ASB17* was high in this population. The inbreeding detected in Caspian horse population may be as a result of diminished population size with an insufficient number of breeding males in the breeding region. However, high levels of heterozygosity, PIC and moderate level of inbreeding in Caspian horse population showed

Table 3. Results of the bottleneck detection tests on the Caspian horse

Test/model	IAM	TPM	SMM
Sign test: number of loci with heterozygosity excess: expected value (probability)	9.88 (0.010*)	9.55 (0.048*)	8.57 (0.002*)
Standard difference test: Ti value (probability)	3.125 (0.002)*	0.452 (0.255)	1.112 (0.078)
Wilcoxon rank test (probability of heterozygosity excess)	0.001*	0.048*	0.989

a high genetic variability that can be employed by horse breeders for planning breeding strategies and focus on the breed for its conservation. The International Stud Book Committee (ISBC) has recommended that the combined exclusion probability (CPE) value for parentage verification in a horse be higher than 0.9995 (Tozaki *et al*., 2001). In this study, the CPE using 17 microsatellite markers was greater than the value required by the ISBC. Other studies reported similar values of combined exclusion probability (0.999) in Thoroughbred and Arabian horse (Khanshour and Cothran, 2012; Cho, 2006; Lee and Cho, 2006). Ellegren *et al*. (1992) proposed at least ten microsatellite loci should be used to gain maximum exclusion in horses. Marklund *et al*. (1994) analyzed eight microsatellite loci in parentage testing to gain a combined exclusion probability of 0.96 to 0.99 in different breeds. At least five microsatellite loci with PE more than 97% should be used to obtain a high degree of excluding probability (Jakabova *et al*., 2002). Rahimi Mianjia *et al*. (2015) also reported a total PE of 0.993 for twelve microsatellite loci used in Turkmen horse parentage control. These several results comparison with our results shows that our selected microsatellites have greater power of exclusion. The prosperity of paternity testing is not only depends on the number of loci but on the level of informativeness that these markers provide. The level of informativeness of a microsatellite marker is specified by its values of heterozygosity, PIC, PE and genetic diversity and these values are dependent on the number and frequency of alleles in the population (Curi and Lopes, 2002). These values obtained for microsatellite markers used in this study indicated the high level of informativeness of these markers in Caspian horse population. So, these microsatellite markers, showed to be adequate to parentage verification and for individual identification in Caspian horse. Our data showed decrepitude in the individual identification system and confirmed interest in using genetic markers in this system. Identification and parentage verification of the Caspian horse population using a panel of microsatellite markers would be very important for the conservation program of this breed. When a population goes through a bottleneck, rare alleles tend to be lost and allelic diversity or, average number of alleles per locus, is reduced. However, heterozygosity is not reduced proportionally, because rare alleles contribute little to heterozygosity (Pandey *et al*., 2006). Cornuet and Luikart, (1998) described populations showing a signiûcant heterozygosity excess would be considered as having experienced a recent bottleneck. Mutations in microsatellites generally do not appear consistently with either the IAM or the SMM. The TPM model is better for most of the microsatellites and the Wilcoxon sign-rank test shows the highest statistical power (Luikart *et al*., 1998). Therefore, Wilcoxon rank test results should be considered to be more trustworthy for this study, and revealed the bottleneck events. In this study, we propose that the Caspian horse population has decreased to low numbers in the past, but a bottleneck event is still scrutable, and its number has recently increased. Detection of bottlenecked populations is important for design of conservation strategies. Usually those populations suffer from inbreeding, decrease of genetic variation, ûxation of detrimental alleles and these factors can reduce the adaptive potential or the probability of population persistence.

CONCLUSIONS

The present research contributes to the knowledge of genetic structure and estimation of existing genetic diversity in the Caspian horse population. Hopefully this study will be useful in conservation plans and breeding strategies.

SUGGESTION

Further genetic studies of the other Iranian horse breeds need to be accomplish to determine the phylogenic relationships among the indigenous horse breeds.

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