Genetic Characterization and Bottleneck Demographic Assessment of Caspian Horse Population

KARAKTERISASI GENETIK DAN HAMBATAN DEMOGRAFI PADA PENILAIAN POPULASI KUDA KASPIA

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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 (rataannya 0.684), and 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. The total exclusion probability of 17 microsatellite loci was 0.9999. The low values of Wright’s fixation index/ Fis (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 horse population and helps to define its genetic conservation strategies.

Keywords: genetic characterization; microsatellite markers; Caspian horse

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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 horses 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 ūrst 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).

RESEARCH 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 MgCl₂ (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
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 (HO), expected heterozygosity (He), Polymorphic information content (PIC) and combined probability of exclusion (PE). Deviations from HWE and inbreeding coefficient (Fis) 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 (Fis) 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, 9.4. An average of 0.084 was recorded the within population inbreeding coefficient (Fis).

Table 2. Number of alleles (Na), observed heterozygosity (H0), expected heterozygosity (He), Polymorphic information content (PIC), inbreeding coefficient (Fis), exclusion probabilities (PE) and Hardy Weinberg Equilibrium (HWE) of 17 microsatellites loci for Caspian horse.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Na</th>
<th>H0</th>
<th>He</th>
<th>PIC</th>
<th>Fis</th>
<th>PE</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHT04</td>
<td>8</td>
<td>0.831</td>
<td>0.805</td>
<td>0.834</td>
<td>-0.032</td>
<td>0.685</td>
<td>NS</td>
</tr>
<tr>
<td>AHT05</td>
<td>8</td>
<td>0.712</td>
<td>0.715</td>
<td>0.818</td>
<td>0.004</td>
<td>0.625</td>
<td>NS</td>
</tr>
<tr>
<td>ASB02</td>
<td>8</td>
<td>0.762</td>
<td>0.723</td>
<td>0.752</td>
<td>-0.054</td>
<td>0.645</td>
<td>NS</td>
</tr>
<tr>
<td>ASB17</td>
<td>8</td>
<td>0.505</td>
<td>0.798</td>
<td>0.785</td>
<td>0.367</td>
<td>0.633</td>
<td>**</td>
</tr>
<tr>
<td>ASB23</td>
<td>7</td>
<td>0.625</td>
<td>0.705</td>
<td>0.724</td>
<td>0.113</td>
<td>0.576</td>
<td>**</td>
</tr>
<tr>
<td>HMS01</td>
<td>5</td>
<td>0.512</td>
<td>0.721</td>
<td>0.716</td>
<td>0.290</td>
<td>0.426</td>
<td>**</td>
</tr>
<tr>
<td>HMS02</td>
<td>8</td>
<td>0.712</td>
<td>0.753</td>
<td>0.827</td>
<td>0.054</td>
<td>0.636</td>
<td>*</td>
</tr>
<tr>
<td>HMS03</td>
<td>8</td>
<td>0.824</td>
<td>0.815</td>
<td>0.801</td>
<td>-0.011</td>
<td>0.623</td>
<td>NS</td>
</tr>
<tr>
<td>HMS06</td>
<td>7</td>
<td>0.723</td>
<td>0.781</td>
<td>0.791</td>
<td>0.075</td>
<td>0.553</td>
<td>*</td>
</tr>
<tr>
<td>HMS07</td>
<td>8</td>
<td>0.575</td>
<td>0.835</td>
<td>0.785</td>
<td>0.311</td>
<td>0.633</td>
<td>**</td>
</tr>
<tr>
<td>HTG04</td>
<td>8</td>
<td>0.732</td>
<td>0.715</td>
<td>0.758</td>
<td>-0.023</td>
<td>0.620</td>
<td>NS</td>
</tr>
<tr>
<td>HTG06</td>
<td>7</td>
<td>0.817</td>
<td>0.732</td>
<td>0.804</td>
<td>-0.116</td>
<td>0.531</td>
<td>**</td>
</tr>
<tr>
<td>HTG07</td>
<td>5</td>
<td>0.514</td>
<td>0.711</td>
<td>0.738</td>
<td>0.277</td>
<td>0.429</td>
<td>**</td>
</tr>
<tr>
<td>HTG10</td>
<td>9</td>
<td>0.745</td>
<td>0.615</td>
<td>0.824</td>
<td>-0.177</td>
<td>0.813</td>
<td>**</td>
</tr>
<tr>
<td>LEX33</td>
<td>8</td>
<td>0.682</td>
<td>0.801</td>
<td>0.798</td>
<td>0.148</td>
<td>0.721</td>
<td>**</td>
</tr>
<tr>
<td>UCDEQ425</td>
<td>6</td>
<td>0.612</td>
<td>0.703</td>
<td>0.799</td>
<td>0.130</td>
<td>0.615</td>
<td>**</td>
</tr>
<tr>
<td>VHL20</td>
<td>8</td>
<td>0.754</td>
<td>0.812</td>
<td>0.827</td>
<td>0.071</td>
<td>0.683</td>
<td>*</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>7.41±0.23</td>
<td>0.684±0.05</td>
<td>0.748±0.15</td>
<td>0.787±0.11</td>
<td>0.084±0.04</td>
<td>0.9999</td>
<td>-</td>
</tr>
</tbody>
</table>
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).

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

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

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 Arab 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 Ho than He values in the Hucul horse population. Five (AHT04, AHT05, ASB02, HMS03 and HTG04) of the 17 loci did not show significant 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 Fis 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...
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 that 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 significant 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, fixation 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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