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Research Article

Microsatellite Analysis for Parentage Verification and Genetic Characterization of the Turkmen Horse Population

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Abstract

Microsatellites are a class of genetic markers commonly used for parentage verification and population studies. This study determined the efficiency of microsatellite markers for identification and pedigree analysis in horses based on the example of Turkmen horse population. For this purpose, 748 Turkmen horse samples including 574 adults (92 stallions, 345 males and 137 mares) and 174 foals (98 colts and 76 fillies),were genotyped by using seventeen microsatellite markers recommended by ISAG. The number of allele per locus varied from 5 (HMS01 and HTG07) to 10 (HTG10) with an average value of 7.65. The observed heterozygosity and the expected heterozygosity ranged 0.365-0.953 (mean 0.703), from 0.617-0.884 (mean 0.792) respectively. PIC value ranged from 0.586 (HTG7) to 0.873 (HTG10) with average 0.767. The total exclusion probability of the 17 microsatellite loci was 0.9999. The pedigree study of the Turkmen horse using microsatellite markers was efficient in detecting mistakes during genealogical records. These results suggested that the DNA typing method had high potential for systematic control of the genealogical registrations and genetic resources to improve genetic aspects in Turkmen horses.

Keywords: *Parentage verification, Genetic characterization, Microsatellite markers, Turkmen horse*

Türkmen At Popülasyonunda Soy Tespiti amacıyla Mikrosatellit Analiz ve Genetik Karakterizasyon

Özet

Mikrosatellitler yaygın olarak soy tespiti amacıyla ve popülasyon çalışmalarında kullanılan bir sınıf genetik belirteçlerdir. Bu çalışma, Türkmen at popülasyonunda identifikasyon ve soy analizinde mikrosatellit belirteçlerin kullanılabilirliğini tespit etmek amacıyla yapılmıştır. Bu amaçla, 574 ergin (92 aygır, 345 beygir ve 137 kısrak) ve 174 tay (98 erkek tay ve 76 dişi tay) içeren toplam 748 Türkmen ata ait örnekler ISAG tarafından önerilen 17 mikrosatellit belirteç kullanılarak genotiplendirildi. Her bir lokusta allel sayısı 5 (HMS01 ve HTG07) ile 10 (HTG10) arasında olmak üzere ortalama 7.65 olarak tespit edildi. Gözlemlenen heterozigotluk ve beklenen heterozigotluk sırasıyla 0.365-0.953 (ortalama 0.703) ve 0.617-0.884 (ortalama 0.792) olarak belirlendi. Polimorfizm bilgi içeriği ortalama 0.767 olmak üzere 0.586 (HTG7) ile 0.873 (HTG10) arasında değişim gösterdi. 17 mikrosatellit bölgenin total dışlama olasılığı 0.9999 idi. Mikrosatellit belirteçler kullanılarak yapılan Türkmen atlarındaki soy araştırması soy kayıtlarındaki hataları tespit etmede etkiliydi. DNA tiplendirme metodu soy kayıtlarının sistemik kontrolünde yüksek potansiyele sahip olup Türkmen atlarının genetik kaynağını artırmada kullanılabilir.

Anahtar sözcükler: *Soy tespiti, Genetik karakterizasyon, Mikrosatellit belirteçler, Türkmen atı*

INTRODUCTION

Horses are belonging to Equidae family; the horse's influence on human history and civilization make it one of the most important animals [1]. Iran has a long history of horse domestication and breeding [2]. Iranian horse breeds may be classified into 4 main groups according to their origins and habitats: North alluvial plains such as Caspian breed, northeast fields such as Turkmen breed, and west highlands such as Kurd breed and southwest and central plateau such as Persian-Arab breed [2]. Turkmen horse is one of the oldest breeds in the world and always achieves high ranks in courses and jumping competitions [3]. Studbook data includes some errors in the registration of the Turkmen studbook. Those data important to the conservation of the breed and correct lines of ancestral might be essential for breeding of Turkmen horse. This information might be verified by a molecular data. It

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is necessary, because the genetic characterization of a breed is the first step in the conservation of breeds, determination of future breeding strategies, and is important to protect breed integrity [4]. Identification of genetic variation among various horse breeds requires the development of genetic markers [5]. Microsatellite markers are useful to diversity studies in the animal. They are tandemly repeated sequences which can be genotyped by PCR techniques [6]. Even in the time of wide genome scanning with the use of SNP microarrays, microsatellites are still used in the construction of linkage maps, when narrowing down the regions of QTLs [7]. Microsatellite markers were first characterized in Swedish horse [8,9]. Commonly, in horses set of seventeen basic microsatellites loci are used (Equine Genetics and Parentage Analysis Workshop, 2012). This horse genotyping panel has been designed to provide high discrimination power (PD), with minimum time need to sample preparation and minimum use of reagents [10]. Those markers constitute a panel of loci recommended by International Society for Animal Genetics (ISAG) in horses parentage testing. In Iran, the polymorphism of these markers has been proved to be useful in Caspian horse [11]; Kurd [12] and Iranian-Arab horse [13]. Application of microsatellite markers in evaluation of the genetic structure in Turkmen horses has not been done yet and this is the first research for parentage verifications based on seventeen microsatellites loci recommended by ISAG's in this breed. The purpose of this study was to evaluate these microsatellite loci in Turkmen horse population and design a marker system for future low-cost genotyping, which will give high combined exclusion probabilities (EPs).

MATERIALS and METHODS

Animals and DNA Extraction

The animals were randomly chosen by their breeders who were able to document their pedigrees (parents, offspring). Blood samples were collected from 748 Turkmen horses, 574 adults (92 stallions, 345 males and 137 mares) and 174 foals (98 colts and 76 fillies). Genomic DNA was extracted from blood samples using the salting-out method [14].

Microsatellite Markers Genotyping

Seventeen microsatellites were selected for this study that had been reported by ISAG for individual identification and parentage verification of Turkmen horses. Microsatellite markers *(Table 1)* were combined in multiplex PCR reaction using fluorescently labeled primers and amplified in a total volume of 20 µl of the following mixture: 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 GeneAmp PCR 9700 (Applied Biosystems, USA). PCR amplification was as follows: the first step was performed by initial denaturation for 5 min at 95°C, followed by 35 cycles at 95°C for 30 sec, 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

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), were computed using CERVUS version 3 software [15]. Deviations from HWE and inbreeding coefficient (F_{is}) were estimated by GENEPOP version 4.4 program [16].

RESULTS

Microsatellite Polymorphism

A total of 130 alleles were observed among the 748 animals and demonstrated that they were highly polymorphic in Turkmen horse populations. A number of alleles, observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphic information content and exclusion probability (PE) in the Turkmen horse were shown in *Table 2*. The number of allele per locus varied from 5 (HMS01 and HTG07) to 10 (HTG10) with an average value of 7.65 in the Turkmen horse. The observed heterozygosity and the expected heterozygosity ranged 0.365-0.953 (mean 0.703), from 0.617-0.884 (mean 0.792) respectively. Microsatellite markers showing PIC values higher than 0.5 are commonly considered as informative in horse population [17]. All marker loci engaged in this study were informative since the average PIC value calculated at 0.767. The lowest PIC value was for HTG7 (0.586), while the highest value was for HTG10 (0.873). The within population inbreeding estimate (F_{is}) ranged between -0.246 and 0.482 with an average of 0.081. Thus, on an average, deficiency (8.1%) of heterozygote existed in the Turkmen horse population *(Table 2)*. Statistically significant deviation from Hardy-Weinberg equilibrium ($P<0.05$) was found at total loci, except (AHT5, ASB17, HTG4, LEX33and UCDEQ425) loci *(Table 2)*. The obtained PE for each polymorphic locus was ranged from 0.3098 for HTG07 to 0.803 for HTG10 with a combined average probability of exclusion of 0.99999 *(Table 2)*. The parentage testing

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

of the 174 foals was verified by the compatibility of 17 microsatellite markers according to Mendelian laws and using likelihood based method. However, 26 foals did not inherit alleles from the registered sire and 9 foals did not inherit alleles from the registered dam.

DISCUSSION

The use of microsatellite markers for individual identification and parentage verification of horses is a routine method in several countries [18]. The present study describes the utility of seventeen microsatellite markers in parentage verification in Turkmen horse breeds. For a clear genetic differentiation between breeds, it has recommended a minimum of four alleles per locus by FAO [19]. Consequently, All 17 microsatellite markers applied in this study showed reliable polymorphism for evaluating genetic variation within the Turkmen horse population. The allele numbers and heterozygosity levels observed this study, indicate a presence of a reasonably high level of genetic variability in Turkmen horse population. The genetic structure of the Turkmen horse population revealed an increased allelic diversity for 17 microsatellites in relation to other studies. With the same set of microsatellite markers, Georgescu et al.[20], investigated the structure of indigenous Romanian Hucul horse breed. The observed and expected heterozygosity per breed ranged from 0.662 and 0.676, respectively. Genetic variation among four Italian horse breeds was assessed using a set of 11 microsatellites [21]. In the breed level, it was showed a high level of gene diversity (He) ranging from 0.71 in Sicilian Oriental Purebred to 0.81 in Sicilian Indigenous [21]. 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 0.767 which is moderate polymorphic. For linkage mapping, Dierks et al.[7], selected microsatellite markers with PIC values >0.5 as markers with values below this level are insufficient for parentage verification. In their study, the

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 Turkmen horse*

Loci	Na	H_O	H_E	PIC	F_{is}	PE	HWE
AHT04	9	0.755	0.825	0.826	0.092	0.701	**
AHT05	8	0.521	0.725	0.799	0.281	0.619	NS
ASB02	9	0.625	0.734	0.825	0.148	0.633	**
ASB17	8	0.452	0.758	0.810	0.403	0.629	NS
ASB23	8	0.852	0.725	0.815	-0.170	0.476	**
HMS01	5	0.425	0.821	0.664	0.482	0.476	**
HMS02	7	0.732	0.789	0.783	0.072	0.605	**
HMS03	9	0.786	0.725	0.829	-0.084	0.655	**
HMS06	7	0.724	0.821	0.730	0.118	0.532	*
HMS07	9	0.701	0.822	0.794	0.166	0.627	*
HTG04	7	0.671	0.727	0.593	0.077	0.310	NS
HTG06	6	0.809	0.712	0.703	-0.136	0.491	**
HTG07	5	0.733	0.726	0.586	-0.009	0.309	**
HTG10	10	0.753	0.604	0.873	-0.246	0.803	**
LEX33	8	0.609	0.793	0.836	0.232	0.701	NS
UCDEQ425	7	0.758	0.723	0.725	-0.048	0.555	NS
VHL20	8	0.794	0.801	0.832	0.008	0.713	*
Mean ± **Sd**	7.65	0.690±0.18	0.731±0.19	0.767±0.15	0.081±0.03	0.99999	-

average PIC value was 0.596 with a maximum of 0.866 [7]. The inbreeding index Fis indicates moderate level of inbreeding in Turkmen horse population, but Fis for locus HMS1 and ASB2 was high in this population. The inbreeding detected in Turkmen horse population may be as a result of depauperate population size, small breeding areas and/or with an insufficient number of breeding males in the breeding region. However, high levels of heterozygousity, PIC and moderate level of inbreeding in Turkmen horse population reflect high genetic variability that can be exploited by horse breeders for planning breeding strategies and prioritizing the breed for its conservation. The International Stud Book Committee (ISBC) has required that the combined exclusion probability (CPE) value for paternity testing and an individual identification in a horse be higher than 0.9995 [22]. In this study, the CPE using 17 microsatellite markers was greater than the value required by the ISBC. Other studies reported similar values of total exclusion probability (0.999) in Thoroughbred and Arabian horse [23-25]. Ellegren et al.[8], proposed at least ten microsatellite loci should be used to gain maximum exclusion in horses. Marklund et al.[9], 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 [26]. Seyedabadi et al.[11], also reported a total PE of 0.973 for seven microsatellite loci used in Caspian horse parentage control. These various 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 [27]. These values obtained for microsatellite markers used in our study indicated the high level of informativeness of these markers in Turkmen horse population. So, these microsatellite markers (ISAG), showed to be adequate to parentage verification and for individual identification in Turkmen 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 Turkmen horse population using a panel of microsatellite markers would be of great importance for the conservation program being applied to this breed.

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