Parentage verification of Iranian Caspian horse using microsatellites markers

Hamidreza Seyedabadi*, Cyrus Amirinia, Mohammad Hossein Banabazi, Hossein Emrani

Department of Biotechnology, Animal Science Research Institute of Iran (ASRI), P.O. Box 1483-31585, *Karaj, I.R. Iran*

Abstract

The present study was to construct a parentage verification system for Iranian Caspian horse. A total number of 45 Caspian horse samples including 14 foals for parentage verification, 17 stallion and 14 mare for individual identification were genotyped. Genomic DNA was extracted from whole blood and the genotype were analysed by PCR procedure and using 7 microsatellite markers (AHT04, HMS03, HMS06, HTG06, HTG07, LEX33 and VHL20). The number of alleles per locus varied from 3 to 4 with mean value of 3.86. The expected heterozygosity was ranged from 0.617 to 0.741 (mean 0.675), and the total exclusion probability (PE) of 7 microstellite loci was 0.973. All markers have relatively high polymorphic information content (PIC) value (> 0.6). All foals were qualified by compatibility according to the Mendelism. This study suggests that the DNA typing method has high potential for parentage testing and individual identification of Iranian Caspian horses.

Keywords: Iranian Caspian horse; Microsatellite; Parentage verification.

INTRODUCTION

The Caspian horse is a beautiful creature with a wonderful temperament. They have beautiful movement which, making them desirable show ponies. Thought to be extinct for 1300 years, Caspians once graced the royal seal of King Darius-550 B.C. Caspians were often depicted in ancient Persian statuettes, friezes and writings going back to 3000 B.C. The Caspian Breed was rediscovered in 1965 (Afraz *et al*., 2005). Domestic and Przewalski's horse (Mongolian wild horse) are belonged to Species of *Equus caballus* and *Equus ferus przewalskii*, respectively. It is suggested that the Caspian horse is the product of natural hybridization between *E. caballus* and *E. prezwalskii* (Hatami and Pandit, 1979). A survey conducted from July of 1965 through August 1968 indicated that there were approximately fifty small horses with definite Caspian characteristics along the entire littoral of the Caspian Sea. Due to the fact that the individuals were widely scattered, it was virtually impossible for any of them to be considered completely pure. The current estimated Iranian registered population of caspian horses is approximately 150 individuals.

Horse breeders provided a horse parentage data to breeding societies, which enter the data into the registry to generate pedigrees. Thoroughbred horse registries have verified pedigree records and resolved queries of parentage using microsatellite DNA typing (Tozaki, 2001). The term microsatellites, also short tandem repeats (STRs), refers to a class of codominant DNA markers which are inheriting a Mendelian fashion This method has become the most effective for pedigree maintenance of large populations of animals because of the decrease in price of reagents and instruments used (Dimsoski, 2003). In cattle, pig and canine, pedigree control has been performed on routine basis in most countries relying on DNA typing that have been standardized through regular comparison tests under the auspices of the International Society for Animal Genetics (ISAG) (Cho *et al*., 2004). International Stud Book Committees (ISBC) has required a higher probability of exclusion (PE) values for parentage verification and an individual identification in horse (Tozaki, 2001). The PE is a parameter to solve problems of some genetic markers in a popula-

*Corresponding Author: **Hamidreza Seyedabadi, Ph.D.**
Tel: +98 261 4466226; Fax: +98 261 4466230
E-mail: h_seyedabadi@asri.ir

tion and is most commonly used as molecular markers in pedigree verification (Luikart *et al*., 1999).

DNA based methods offer several potential advantages compared with conventional parentage testing systems because of their accuracy and specificity. Microsatellites have been chosen as the suitable markers for parentage testing which can be easily scored by a computer program. Microsatellites are highly polymorphic and abundant sequences dispersed throughout most eukaryotic nuclear genomes (Litt and Luty, 1989). Microsatellites are valuable genetic markers due to their dense distribution in the genome, great variation, co-dominant inheritance and easy genotyping. In recent years, they have been extensively used in parentage testing, linkage analyses, population genetics and genetic studies (Goldstein and Pollock, 1997). Many microsatellites are informative due to their high polymorphisms and they are useful in paternity testing of horses such as native horses (Bowling *et al*.,1997). Equine microsatellites were first characterized by Ellegren and cowrker (1992) and Marklund and cowrker (1994) who isolated a set of $(CA)_n$ repeats and demonstrated that they were highly polymorphic in horse. The horse genetic committee of ISAG presented 9 microsatellite markers (AHT04, HMS03, HMS06, HTG06, HTG07, LEX33, ASB2, HTG10 and VHL20) as international minimum standard microsatellite marker system, as well as additional markers (ASB17, ASB23, CA425, HMS1, LEX3, LEX33 and TKY321) to be typed for horse parentage testing (Lee and Cho, 2006).

The aim of the present study is to verifying parentage in Caspian horse population using microsatelite loci to introduce a reliable parentage test. We performed a routine DNA typing with 7 standard microsatellite markers for parentage verification and individual identification of Iranian Caspian horses. Number of alleles, heterozygosities, polymorphic information content (PIC) and exclusion probability (PE) were also calculated.

MATERIALS AND METHODS

Whole blood samples were collected from 45 horses including 14 foals, 17 stallion and 14 mare. Genomic DNAs were extracted using salting-out method with some modifications (Miller *et al*., 1988). Seven microsatellite markers were selected for this study (Table 1). These microsatellite markers have been

Table 1. Characteristics of 7 microsatellite loci used in this study.

Locus name	Chromosome No.	Primer Sequence	Accession numbers	Allele range(bp)	Reference
HMS06	4	F-GAAGCTGCCAGTATTCAACCATTG R-CTCCATCTTGTGAAGTGTAACTCA	X74635	153-169	Guerin *et al*. (1994)
HMS03	9	F-CCAACTCTTTGTCACATAACAAGA R-CCATCCTCACTTTTTCACTTTGTT	X74632	148-186	Guerin *et al*. (1994)
AHT04	21	F-AACCGCCTGAGCAAGGAAGT R-CCCAGAGAGTTTACCCT	None	139-171	Binns *et al*. (1995)
HTG06	15	F-CCTGCTTGGAGGCTGTGATAAGAT R-GTTCACTGAATGTCAAATTCTGCT	None	85-112	Ellegren *et al*. (1992)
HTG07	4	F-CCTGAAGCAGAACATCCCTCCTTG R- ATAAAGTGTCTGGGCAGAGCTGCT	None	120-130	Ellegren *et al*. (1992)
LEX33	4	F-TTTAATCAAAGGATTCAGTTG R-TTTCTCTTCAGGTGTCCTC	AF075635	203-217	Coogle *et al*. (1996)
VHL20	30	F-CAAGTCCTCTTACTTGAAGACTAG R-AACTCAGGGAGAATCTTCCTCAG	None	89-107	van Haeringen *et al*. (1994)

reported by the horse applied genetics committee of ISAG for individual identification and parentage verification. Fifteen µl of PCR mixture containing 1X PCR buffer; 5 mM $MgCl_2$; 0.25 µM of each primer; dNTPs 200 µM; 1 Unit of *Taq* DNA polymerase and 150 ng of genomic DNA were used for amplification. PCR conditions for all loci were included initial denaturation at 95ºC for 2.5 min followed by 36 cycles of 95ºC for 30s;annealing at 60ºC for 30s; extension at 72ºC for 45s; which followed for one cycle at 72ºC for 5 min as final extention. The PCR products were electrophoresed on 8% nondenaturing polyacrylamide gels for overnight and the bands visualized by rapid silver staining (Sanguinetti *et al*., 1994). Parentage verification was performed according to Mendelian fashion and ISAG guidelines. Allelic frequencies and number of alleles per locus were estimated by direct counting from observed genotypes. Heterozygosities, polymorphic information contents and exclusion probabilities were computed using the CERVUS (Ver. 2.0) software (Marshall *et al*., 1998).

RESULTS

The results of DNA typing for parentage testing in 14 foals were verified by the compatiability of 7 microsatellite markers according to Mendelian inheritence pattern. Figure 1 shows one example of verification and mendelian inheritence pattern using AHT04 locus. Microsatellites were polymorphic in Caspian horses (Table 2 and 3). All of the primers amplified the needed fragments very well. The average number of alleles was 3.86 which ranged from 3 to 4 per locus. The highest observed allel frequency in each locus was

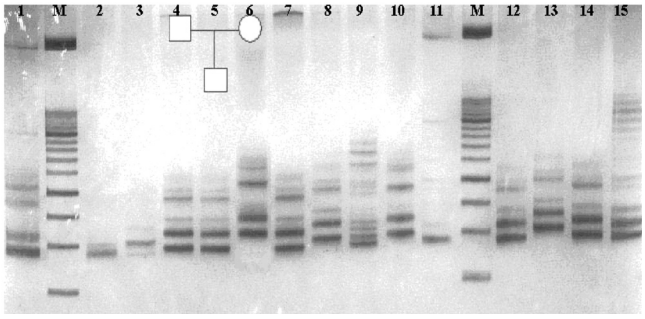

Figure 1. An example of parentage verification in one Iranian Caspian horse foal using AHT04 locus. Lanes from 1 to 15 are individuals including parents and their offsprings. Individuals 4 and 6 are dam and sire for 5, respectively. Mendelian inheritance has been qualified. M is size marker XIII of Roche company-Germany.

0.3194 for HMS06 (167 and 169 bp), 0.3333 for AHT04 (139 bp), 0.4778 for HTG06 (85 bp), 0.500 for HMS03 (182 bp), 0.500 for HTG07 (123bp), 0.3750 for LEX 33 (205 and 217 bp) and 0.4048 for VHL20 (92 and 107bp). The observed heterozygosity (H_0), expected heterozygosity (H_e), polymorphic information content (PIC) and exclusion probability (PE) are shown in Table 3. The observed heterozygosity (H_0) and expected heterozygosity ranged from 0.756 to 1 (the average value was 0.946) and from 0.617 to 0.741 (the average value was 0.675), respectively. PIC values ranged from 0.53 to 0.681 with a mean value of 0.605 and PE values ranged from 0.321 to 0.480. The total exclusion probability (PE) of 7 microstellite loci was 0.973 in Caspian horse population.

DISCUSSION

Horse breed registry societies rely on genetic testing as part of the process to ensure pedigree integrity. Equine

Table 2. Allele frequencies of microsatellite loci in Caspian horse.

Locus name	No. of alleles	Allele Size (frequency)
HMS06	4	153bp(0.1806),155bp(0.1806),167bp(0.3194),169bp(0.3194)
HMS03	4	148bp(0.0667),150bp(0.2333),182bp(0.5000),186bp(0.2000)
AHT04	4	164bp(0.3333),166bp(0.3222),169bp(0.2667),171bp(0.0778)
HTG06	4	85bp(0.4778),88bp(0.0889),110bp(0.3667),112bp(0.0667)
HTG07	3	120bp(0.1563),123bp(0.5000),130bp(0.3438)
LEX33	4	203bp(0.1250),205bp(0.3750),215bp(0.1250),217bp(0.3750)
VHL20	4	89bp(0.0952),92bp(0.4048),105bp(0.0952),107bp(0.4048)

Table 3. Heterozygosity, PIC value and PE of microsatellite markers used in Caspian horse.

Locus name	No. of allele	H_O	H_e	PIC	PE
HMS06	4	1.000	0.741	0.681	0.480
HMS03	4	1.000	0.658	0.596	0.395
AHT04	4	0.756	0.716	0.651	0.444
HTG06	4	0.867	0.632	0.555	0.353
HTG07	3	1.000	0.662	0.588	0.385
LEX33	4	1.000	0.617	0.530	0.321
VHL20	4	1.000	0.698	0.630	0.427
Mean	3.86	0.940	0.675	0.605	-
Total exclusion probability					0.973

*Combined exclusion probability.

blood typing and microsatellite DNA typing are now indispensable for the accurate recording of horses worldwide (Bowling *et al*., 1997). Use of microsatellite genotyping for individual identification, parentage control and solving problems of questionable maternity or paternity is a routine procedure within the horse breeding industry in several countries (Siegal, 1996). The high polymorphism with well balanced frequencies of the alleles makes these systems attractive for parentage control. They are not only highly informative but also quite simple to use and standardise. The genetic analysis using the seven microsatellite loci has revealed that they have a total exclusion probability (Weir, 1996) of 97.3%. The horse genetic committee of ISAG has recommended that parentage testing should consist of an exclusion based on the incompatibility of two or more markers, because an exclusion based on a single marker may involve an element of uncertainty. All possibilities should be tried to obtain additional information to support a decision for such an exclusion, including tests for additional markers or mutation analysis (Binns *et al*., 1995).

Jakabova and collegues (2002) have also shown that at least five microsatellites with the highest individual PE values that have a 97% total exclusion probability should be used to obtain a high degree of excluding incorrect parentage. Usha *et al*. in 1994 also reported a total PE of 0.88 for two microsatellite loci used in cattle parentage control. Ellegren and collegues in 1992 suggested that at least ten microsatellite loci should be used to achieve a maximum exclusion in horses. Marklund and co workers (1994) analysed eight microsatellite loci in paternity testing to reach a total exclusion probability of 0.96–0.99 in different breeds. Comparison of our results with these various results clearly shows that our selected microsatellites have greater power of exclusion given the fact that we could reach a high level of exclusion with only seven loci (PE = 0.973). Analyses of more loci will allow to increase the combination efficiency.

We constructed a paternity testing system for individual identification and parentage verification of the Caspian horse and investigated a validation of 7 microsatellite markers for routine parentage testing and polymorphisms in this population. Our estimate (H_0 = 0.94) of genetic diversity shows a higher level of diversity than that (H_0= 0.42) reported by Shahsavarani *et al*., 2006. The difference of the genetic diversity value surveyed could probably be explained by the choice of the microsatellite markers. The average expected heterozygosity (0.675) indicated the existence of high genetic variability within the Caspian horse population. PIC values ranged from 0.530 to 0.681 with mean value of 0.605 in terms of their suitability for genetic diversity and paternity studies, and the remaining loci were reasonably informative (Table 3). In this study, PE value (PE= 0.973) is lower than Thoroughbred horse (PE= 0.9979) reported by Cho (2002) and Korian Thoroughbred horse (PE= 0.9998) reported by Lee and Cho (2006). All foals selected for parentage testing by DNA typing, were qualified by compatibility of 7 markers according to Mendelian inheritence pattern. These results suggest that the present DNA typing has a particial efficacy for the Caspian horse parentage verification.

We can conclude that selection of the microsatellites is important and effective in resolving parentage testing. This 7 microsatellite markers system is considered to be greatly useful for parentage verification on Caspian horse. Results can give basic information for developing accurate parentage verification and individual identification system in the Iranian Caspian horse.

Acknowledgments

Authors thank A. Javanrouh, N. Asadi and S. Dordari for technical assistance and M.A. Kamali for his comments on the manuscript.

References

Afraz F, Esmaeelkhanian S, Seyedabadi HR (2005). Investigation on blood protein polymorphisms of Arab and Caspian miniature horses in IRAN. *J Pajouhesh Sazandegi* (In Farsi). 66: 33-38.

Binns M, Uolmes N, Holliman A (1995). The identification of polymorphic microsatellite loci in the horse and their use in thoroughbred parentage testing. *Brit Vet J*. 151: 9-15.

Bowling AT, Eggleston-Stott ML, Byrns G, Clark RS, Dileanis S, Wictum E (1997). Validation of microsatellite markers for routine horse parentage testing. *Anim Genet*. 28: 247-252.

Cho GJ, Cho BW (2004). Microsatellite DNA typing using 16 markers for parentage verification of the Korean native horse. Asian-Aust. *J Anim Sci.* 17: 750-754.

Cho GJ (2002). Microsatellite DNA polymorphism of Thoroughbred horses in Korea. *Koean J Genet*. 24: 177-182.

Coogle L, Bailey E, Reid R, Russ M (1996). Equine dinucleotide repeat polymorphisms at loci LEX002, -003, -004, -005, -007, -008, -009, -010,-011,-013 and -014. *Anim Genet*. 27: 126-127.

Dimsoski P (2003) Development of a 17-plex microsatellite polymerase chain reaction kit for genotyping horses. *Croat Med J*. 44: 332-335.

Ellegren H, Johansson M, Sandberg K, Andersson L (1992). Cloning of highly polymorphic microsatellites in the horse. *Anim Genet*. 23: 133-142.

Goldstein DB, Pollock DD (1997). Launching microsatellttes: a review of mutation. processes and methods of phylogenetic inference. *J Hered*. 88: 335-342.

Guerin G, Bertaud M, Amigues Y (1994). Characterization of seven new horse microsatellites: HMS1, HMS2, HMS3, HMS5, HMS6, HMS7 and HMS8. *Anim Genet*. 25: 62.

Hatami H, Pandit RV (1979). A cytogenetic study of the Caspian pony. *J Reprod Fertil*. 57:331-3.

Jakabova D, Trandzik J, Chrastina J (2002). Effectiveness of six highly polymorphic microsatellite markers in resolving paternity cases in Thoroughbred horses in Slovakia. Czech. *J Anim Sci.* 47: 497-501

Lee SY, Cho GJ (2006). Parentage testing of Thoroughbred horse in Korea using microsatellite DNA typing. *J Vet Sci*. 7: 63-67.

Litt M, Luty JA (1989). A hyper variable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am J Hum Genet*. 44: 397-401.

Luikart G, Biju-Duval MP, Ertugrul Y, Zagdsuren C, Maudet C, Taberlet P (1999). Power of 22 microsatellite markers in fluorescent multiplexes for parentage testing in goats (*Capra hircus*). *Anim Genet*. 30: 431-438.

Marklund S, Ellegren H, Eriksson S, Sandberg K, Andersson L (1994). Parentage testing and linkage analysis in the horse using a set of highly polymorphic microsatellites. *Anim Genet*. 25: 19-23.

Marshall TC, Slate J, Kruuk L, Pemberton JM (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Mol Ecol*. 7: 639-655.

Miller SA, Dykes DD, Polesky HF (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16: 1215.

Sanguinetti CJ, Neto ED, Simpson AJG (1994). Rapid silver staining and recovery of PCR product separated on polyacrylamide gels. *Biotechniques* 17: 915-919.

Shahsavarani H, Rahimi G, Tozaki T, Sayyahzadeh H, Taghavi MJ, Dehestani A, Dordari S (2006). *Using Microsatellite markers to estimate genetic variability in the Caspian horse breed*. 8th World Congress on Genetics Applied to Livestock Production. August 13 to 18. Belo Horizonte. MG. Brazil.

Siegal M (1996). UC Davis Book of Horses*: A Complete Medical Reference Guide for Horses and Foals*. Haper Collins, New York. P: 126-134.

Tozaki T (2001). Characterization of equine microsatellites and repetitive elements, and validation of paternity testing for Thoroughbreds. *Ph.D. Dissertation*. Showa University. Japan.

Usha AP, Simpson SP, Williams JL (1994). Evaluation of microsatellite markers for parentage verification. In: Proceedings of the 24th ISAG Conference. *Anim Genet*. 25: 41.

Van Haeringen H, Bowling AT, Stott ML, Lenstra JA, Zwaagstra KA (1994). A highly polymorphic horse microsatellite locus: VHL20. *Anim Genet*. 25: 207.

Weir BS (1996). *Genetic Data Analysis II.* Sinauer Associates, Inc. Publishers Sunderland, Massachusetts. P: 228-230.