Journal of Animal and Veterinary Advances 11 (15): 2647-2651, 2012
ISSN: 1680-5593

Individual Indentification and Parentage Verification of Thoroughbred Horses and the Korean Native Horses Based on Microsatellite Loci in Korea

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Abstract: This study was performed for individual identification and parentage verification of Thoroughbred Horse (TBH) and the Korean Native Horse (KNH). A total number of 1,308 (84 KNH and 1,224 TBH samples including 1,005 foals for parentage testing) were genotyped. Genomic DNAs were extracted from whole blood or hair roots and genotyped by using 14 microsatellite markers (AHT4, AHT5, ASB2, ASB17, ASB23, CA425, HMS1, HMS3, HMS6, HMS7, HTG4, HTG10, LEX3 and VHL20) in TBH and 17 microsatellite markers including HMS2, HTG6, HTG7 in the KNH. This method consisted of multiplexing PCR procedure and showed reasonable amplification of all PCR products. Genotyping were determined with an ABI 3100 genetic analyzer. All the loci analyzed in 17 microsatellite markers showed a polymorphic patterns. The number of alleles per locus varied from 3-9 with a mean value of 6.36 and 5-10 (mean 7.35) in TBH and KNH, respectively. The expected heterozygosity was ranged from 0.539-0.827 (mean 0.700), from 0.387-0.841 (mean 0.702) in TBH and the KNH, respectively. The total exclusion probability of 14 microsatellite loci was 0.9998 in TBH and 0.9999 in the KNH. Of the 14-17 markers, ASB2, ASB17, ASB23, HMS7, HTG10 and LEX3 loci in TBH and AHT4, AHT5, CA425, HMS2, HMS3, HTG10 and LEX3 loci in the KNH have relatively high PIC value (>0.7). Of the 1005 foals, 1003 foals (99.80%) were qualified by compatibility according to the Mendelian fashion. These results indicated that the present microsatellites serve as useful tools for individual identification and paternity testing of TBH and the KNH in Korea.

Key words: Genotype, horse, microsatellite, parentage verification, products, Korea

INTRODUCTION

Thoroughbred Horse (TBH) is one breed of light horses improved for a purpose of horse racing in the United Kingdom. Korea mainly imported TBH or Anglo-Arab horses from various countries such as Japan and Australia in 1970s for the purpose of racing. In a move to achieve improvement in horse breeding and stud management and eventually contribute to the development of the horse racing industry and the livestock industry, Korea began breeding horses in 1980's. Recently, there are around 2,500 mares and 120 stallions in Korea. These horses give birth to >1,300 foals every year and the number of foals are steadily increasing. Korea Racing Association has played a role the sole authority for TBH registration in Korea since 1993 and published the Korean studbook in 1998. TBH registries have verified pedigree records and resolved queries of parentage using microsatellite DNA typing (Tozaki *et al*., 2001).

The Korean Native Horse (KNH) is one of the Korean native animals which was designated as a natural monument No. 347 by government. At the present, the KNH are raised about 2,000 herds which were finished the pedigree registration in Jeju. It has been insulated from other horse populations and partly has been used racing horse in Jeju race course of Korea Racing Association. Detailed information on levels of genetic diversity and patterns of gene structure of the KNH is very important for meeting the demands of future breeding programs and for formulating effective conversation strategies of indigenous breeds.

In practice, horse breeders provide a horse parentage data to breeding societies which enter the data into the registry to generate pedigrees. The most reliable and efficient method for pedigree construction and analysis is the one that employs the DNA genotyping technology. At the present, the DNA genotyping has become the most effective method for pedigree maintenance of large populations of animals because of the decrease in price of

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reagents and instruments (Dimsoski, 2003). The term microsatellites, also Short Tandem Repeats (STRs) refers to a class of codominant DNA markers which are inherited in a Mendelian fashion. Microsatellites are highly polymorphic and abundant sequences dispersed throughout most eukaryotic nuclear genomes (Litt and Luty, 1989; Weber and May, 1989). Microsatellites have a simple and stable inheritance when they are transmitted from one generation to the next and are controlled only by heredity. Also due to its small size, they are efficiently amplified using PCR techniques. Thus, microsatellites have been used for parentage testing and individual identification in forensic sciences. Many microsatellites are informative due to their high polymorphisms and they are useful in paternity testing of horse such as native horse (Bowling *et al*., 1997). Also microsatellite markers have proven useful in assessing genetic diversity of populations in different species (Xiang-Long and Valentini, 2004).

In cattle (Glowatzki-Mullis *et al*., 1995), pig (Putnova *et al*., 2003) and canine (Cho and Cho, 2003), 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 and Cho, 2004).

In this study, we were performed a routine DNA typing with 14-17 microsatellite markers including 9 international minimum standard microsatellite markers for parentage verification and individual identification of TBH and the KNH. Number of allele, heterozygosities, Polymorphic Information Contents (PIC) and exclusion Probabilities (PE) were calculated.

MATERIALS AND METHODS

Animals and DNA extraction: Genomic DNAs were prepared from whole blood or hair roots samples which were collected from 1,224 TBH including 1,005 foals and 84 KNH. Genomic DNAs from samples were extracted using MagExtractor System MFX-2000 (Toyobo, Japan) according to the manufacturer's protocols (Tozaki *et al*., 2001).

Microsatellite, PCR condition, fragment analysis and parentage testing: The 17 microsatellite markers chosen for this study were: AHT4, AHT5, ASB2, ASB17, ASB23, CA425, HMS1, HMS2, HMS3, HMS6, HMS7, HTG4, HTG6, HTG7, HTG10, LEX3 and VHL20. These microsatellite markers have been reported by the horse applied genetics committee of ISAG for individual identification and parentage verification of horse. Microsatellite markers were combined in multiplex PCR reaction using fluorescently labelled primers and amplified in a total volume of 15 μL of the following mixture: 40 ng of genomic DNA, primer mix, 1.25 mM of dNTPs, 2.5 μL of 10× reaction buffer and 5 U of Taq DNA polymerase (Applied Biosystems, USA). PCR amplification was as follows: 1st step was performed by initial denaturation for 10 min at 95°C, followed by 30 cycles at 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min. An extension step of 72°C for 60 min was added after the final cycle (Dimsoski, 2003). Multiplex PCRs were performed in a GeneAmp PCR System 9700 (Applied Biosystems, USA). PCR products were denatured with formamide and electrophoresis was carried out on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA) using the recommended protocols. Size analyses of DNA fragments separated were performed with genotype software Ver.3.7 (Applied Biosystems, USA). The internal size standard Genescan-LIZ 500 (Applied Biosystems, USA) was used for sizing alleles. In addition, sample No. 1 from ISAG 2005/2006 horse comparison test was used as reference to standardize allele sizes. Parentage testing was performed according to Mendelian fashion and ISAG guideline in the present DNA typing.

Statistical analysis: Allelic frequencies, the number of alleles per locus were estimated by direct counting from observed genotype, heterozygosities, Polymorphic Information Contents (PIC) and exclusion Probabilities (PE) were computed using the CERVEX software (Marshall *et al*., 1998).

RESULTS AND DISCUSSION

Heterozygosities and the number of alleles in TBH: The results of analyses in TBH are shown in Table 1 and 2. The number of alleles varied from 3 (HMS1) to 9 (ASB2, ASB17, HTG10) and the average number of alleles was 6.36 in this study. The observed heterozygosity and expected heterozygosity ranged from 0.456-0.840 (mean 0.682), from 0.539-0.827 (mean 0.700), respectively. PIC value ranged from 0.441-0.805 with a mean value 0.653. Of the 14 markers, ASB2, ASB17, ASB23, HMS7, HTG10 and LEX3 loci have relatively high PIC value (>0.7). The total PE value of 14 microsatellite loci was 0.9998 in TBH.

Heterozygosities and the number of alleles in KNH: The results of analyses in the KNH are shown in Table 3. The number of alleles varied from 5 (HTG4, HTG7) to 10 (ASB17, HTG10, LEX3) and the average number of alleles was 7.35 in this study. The observed heterozygosity and expected heterozygosity ranged from 0.429-0.905 (mean

Table 1: Allele frequencies of microsatellite DNA polymorphisms in 1,224 Thoroughbred horses

Locus	No. of allele	Allele* (frequency)								
AHT4	5	H (0.1818)	J (0.2210)	K (0.2010)	N (0.0004)	O (0.3958)	-	-	-	-
AHT5	5	J (0.1573)	K (0.4910)	M (0.2087)	N (0.1181)	O (0.0249)	-	-	-	-
ASB2	9	B (0.0319)	K (0.1225)	L (0.0004)	M (0.1422)	N (0.1430)	O (0.0903)	P (0.0127)	Q (0.2708)	R (0.1863)
ASB17	9	G (0.3562)	H (0.0020)	I (0.0004)	M (0.0339)	N (0.2345)	O (0.2230)	P (0.0004)	Q (0.0004)	R (0.1491)
ASB23	6	I (0.0658)	J (0.2798)	K (0.2639)	L (0.1920)	S (0.1801)	U (0.0184)	-	-	-
CA425	8	F (0.0004)	I (0.0327)	J (0.1863)	K (0.0020)	L (0.0098)	M (0.0257)	N (0.6397)	O (0.1033)	-
HMS1	3	I (0.1744)	J (0.4555)	M (0.3701)	-	-	-	-	-	-
HMS3	8	I (0.5445)	K (0.0004)	M (0.1471)	N (0.0237)	O (0.1242)	P (0.1467)	Q (0.0004)	R (0.0131)	-
HMS6	5	K (0.1360)	L (0.0290)	M (0.3227)	O (0.0094)	P (0.5029)	-	-	-	-
HMS7	5	J (0.0743)	L (0.1373)	M (0.2426)	N (0.2312)	O (0.3145)	-	-	-	-
HTG4	5	K (0.5453)	L (0.0008)	M (0.4032)	N (0.0208)	P (0.0298)	-	-	-	-
HTG10	9	I (0.2623)	J (0.0004)	K (0.1270)	L (0.1691)	M (0.1953)	N (0.0004)	O (0.1009)	R (0.1438)	S (0.0008)
LEX3	7	F (0.0037)	H (0.3297)	L (0.0229)	M (0.1532)	N (0.1450)	O (0.0588)	P (0.2868)	-	-
VHL20	5	I (0.2672)	L (0.2271)	M (0.3403)	N (0.1626)	O (0.0029)	-	-	-	-

*Alphabetical allele codes for all loci are identical to the assignment on 2000 ISAG horse comparison test

Table 2: Heterozygosity, PIC value and PE of 14 microsatellite markers in 1,224 Thoroughbred horses

Locus	No. of allele	OH_{et}	EH_{et}	PIC	PE*
AHT4	5	0.729	0.721	0.673	0.473
AHT5	5	0.703	0.676	0.632	0.439
ASB2	9	0.840	0.827	0.805	0.657
ASB17	9	0.748	0.745	0.702	0.511
ASB23	6	0.760	0.778	0.742	0.563
CA425	8	0.547	0.544	0.504	0.324
HMS1	3	0.641	0.625	0.547	0.334
HMS3	8	0.609	0.644	0.607	0.420
HMS6	5	0.622	0.624	0.557	0.355
HMS7	5	0.790	0.765	0.725	0.540
HTG4	5	0.544	0.539	0.441	0.248
HTG10	9	0.828	0.818	0.792	0.635
LEX3	7	0.456	0.761	0.723	0.542
VHL20	5	0.735	0.735	0.686	0.487
Mean	6.36	0.682	0.700	0.653	0.9998**

*OH_{et}: Observed Heterozygosity, EH : Expected Heterozygosity, PIC: Polymorphic Information Contents, PE: Exclusion Probability; **Total exclusion probability

Table 3: Heterozygosity, PIC value and PE of 17 microsatellite markers in 84 Korean native horses

Locus	No. of allele	OH_{et}	EH_{et}	PIC	PE*
AHT4	9.00	0.798	0.803	0.770	0.607
AHT5	7.00	0.821	0.742	0.703	0.524
ASB2	7.00	0.560	0.629	0.571	0.380
ASB17	10.00	0.762	0.739	0.693	0.506
ASB23	8.00	0.512	0.713	0.664	0.478
CA425	8.00	0.774	0.753	0.711	0.530
HMS1	6.00	0.607	0.646	0.577	0.378
HMS2	6.00	0.869	0.774	0.734	0.554
HMS3	7.00	0.821	0.795	0.763	0.600
HMS6	6.00	0.810	0.704	0.655	0.464
HMS7	6.00	0.571	0.517	0.490	0.322
HTG4	5.00	0.643	0.596	0.526	0.331
HTG6	6.00	0.429	0.387	0.354	0.205
HTG7	5.00	0.726	0.707	0.665	0.479
HTG10	10.00	0.905	0.825	0.797	0.649
LEX3	10.00	0.464	0.841	0.816	0.676
VHL20	9.00	0.881	0.754	0.720	0.548
Mean	7.35	0.703	0.702	0.659	0.9999**

*OH_{et}: Observed Heterozygosity, EH_{et}: Expected Heterozygosity, PIC: Polymorphic Information Contents, PE: Exclusion Probability; **Total exclusion probability

0.703) from 0.387-0.841 (mean 0.702), respectively. PIC value ranged from 0.354-0.816 with a mean value 0.659. Of the 17 markers, AHT4, AHT5, CA425, HMS2, HMS3, HTG10 and LEX3 loci have relatively high PIC value (>0.7). The total PE value of 17 microsatellite loci was 0.9999 in the KNH.

Parentage verification in TBH: The results of DNA typing for parentage testing in the 2 foals are shown in Table 4. About 2 foals were not inherited alleles from sire or dam and excluded by the incompatibility of 6 and 8 markers, respectively. Of the 1,005 foals, 1,003 foals (99.80%) were qualified by the compatibility of 14 microsatellite markers according to Mendelian fashion in the present DNA typing for parentage verification.

The use of microsatellite typing 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 and Barlough, 1996). The aim of this study was to construct a correct pedigree of TBH and the KNH family. After genotyping, parentage testing was performed according to Mendelian fashion and ISAG guideline.

Equine microsatellites were first characterized by Ellegren *et al.* (1992) and Marklund *et al.* (1994) who isolated set of (CA)n repeats and demonstrated that they were highly polymorphic in horse. 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 markers of choice because of their high levels of polymorphisms which can be easily scored by a computer program. This indicates that DNA typing can be analyzed semi-automatically, alleles of the microsatellites were correctly inherited to the next generation (Tozaki, 2001). International Stud Book Committee (ISBC) has required a higher Probability of Exclusion (PE) value for parentage verification and individual identification in horse, especially TBH (Tozaki *et al.*, 2001). PE is a parameter used to solve the problems of some genetic markers in a population

Table 4: Two cases of parentage testing by 14 microsatellite loci in Thoroughbred horses

		Loci													
Results	Samples	AHT4	AHT5	ASB2	ASB17	ASB23	CA425	HMS1	HMS3	HMS6	HMS7	HTG4	HTG10	LEX3	VHL20
Case 1 (Exclusion)	Sire	H/H	K/N	M/Q	G/R	J/S	N/N	J/M	I/I	K/P	M/O	K/K	I/L	H/H	I/I
	Dam	O/O	K/M	M/Q	N/O	K/L	J/N	J/M	P/P	L/M	L/O	K/K	L/M	H/H	I/M
	Foal	J/J	K/K	M/N	G/N	J/K	N/N	I/M	I/O	M/P	O/O	K/M	I/L	H/H	M/M
Case 2 (Exclusion)	Sire	K/O	K/M	M/O	G/O	J/K	N/O	I/I	I/M	K/M	M/N	K/K	I/K	H/H	I/N
	Dam	H/O	M/M	M/R	N/O	K/S	N/N	M/M	I/M	M/P	M/N	K/P	M/O	M/P	M/N
	Foal	J/K	K/N	M/M	G/O	J/L	N/O	I/J	M/P	K/M	M/O	K/M	K/M	P/P	M/N

*Alphabetical allele codes for all loci are identical to the assignment on 2000 ISAG horse comparison test

(Vegapla *et al*., 1998) and is most commonly used as molecular markers in pedigree verification (Luikart *et al*., 1999).

The Horse Genetic Committee of ISAG presented 9 microsatellite markers (AHT4, AHT5, ASB2, HMS3, HMS6, HMS7, HTG4, HTG10 and VHL20) as international minimum standard microsatellite marker system, as well as additional markers (ASB17, ASB23, CA425, HMS1, HMS2, HTG6, HTG7, LEX3, LEX33 and TKY321) to be typed for horse parentage testing. The Committee 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).

As demonstrated in this study, 1003 foals were qualified by the compatibility of 14 markers according to Mendelian fashion in the present DNA typing for parentage verification. However, 2 foals were not inherited alleles from sire or dam and excluded by the incompatibility of 6 and 8 markers, respectively. The result was in agreement with the previous study that microsatellite DNA typing could be useful for parentage testing. In this study, allelic frequencies provided the combined PE of 0.9998 and 0.9999 on TBH and the KNH, respectively. These are higher than the value (0.9995) of the ISBC proposition (Tozaki *et al*., 2001). The results will be useful to make decisions regarding preservation and further use development of the KNH.

CONCLUSION

Microsatellites have been used for parentage testing and individual identification in forensic sciences. These results testify that 14 or 17 microsatellite markers can be applied very efficiently for resolution of parentage verification and individual identification on TBH and the KNH, respectively.

ACKNOWLEDGEMENTS

This research was carried out with the support of Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ008303) Rural Development Administration, Republic of Korea. Special thanks are due to the staff of Racing Laboratory, Korea Racing Authority for providing materials and their technical assistance.

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