Microsatellite DNA Typing Using 16 Markers for Parentage Verification of the Korean Native Horse

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ABSTRACT : This study was performed for parentage verification of the Korean native horse (KNH). 103 random KNH samples (including 19 foals for parentage testing) were genotyped by using 16 microsatellite markers. The number of alleles per locus varied from 5 to 13 with an average value of 8.56 in the KNH. The observed heterozygosity and the expected heterozygosity ranged 0.398-0.893 (the average value was 0.683) and 0.368-0.871 (the average value was 0.727) in the KNH, respectively. The PIC value and the exclusion probability ranged 0.347-0.853 (the average value was 0.692) and 0.208-0.736, respectively, and the total exclusion probability of 16 microsatellite loci was 0.9999. Of the 16 markers, AHT4, AHT5, ASB2, ASB17, HMS2, HMS3, HTG10, LEX33, TKY321 and VHL20 loci have a relatively high PIC value (>0.7) in the KNH. Of the 19 foals, 5 foals were disqualified by an incompatibility of 4-7 markers according to a Mendelian fashion in the present DNA typing for parentage testing. These results present basic information for developing a system for parentage verification and individual identification in the KNH. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 6 : 750-754)***

Key Words : Allele, Korean Native Horse, Microsatellite DNA, Parentage Verification

INTRODUCTION

The term microsatellites, which are also known as short tandem repeats, refers to a class of codominant DNA markers which are inherited in a Mendelian fashion. Microsatellite loci are widely dispersed along and among choromosomes and each locus is characterized by a known DNA sequence. They are typically composed of between two to four nucleotides such as (CA)n or (GATA)n where *n* lies between 5 and 50 (Dewoody and Avise, 2000). Many kinds of microsatellites are informative due to their high polymorphisms and they are useful in the paternity testing of horses (Bowling et al., 1997). In cattle, pig, horse, goat (Kim et al., 2002) and canine populations, pedigree control has been performed on a routine basis in most countries. These controls rely on a DNA typing that has been standardized through regular comparison tests under the auspices of the International Society for Animal Genetics (ISAG).

The Korean native horse (KNH) is one of the Korean native animals which was designated by the government as a natural monument No. 347 on February 1985 (Cho et al., 2001). They have been separated into distinct areas and partly, they have been used as a racehorse in the Jeju race course of Korea Racing Association. Jeju Island is the major province for producing the Thoroughbred (T.B) in Korea and cross breeding has the potential to eliminate the genetic resource of the KNH by creating a cross breed horse (KNH×Thoroughbred, CBH). Therefore, it is necessary to preserve this genetic resource (Cho et al., 2000b). Detailed information on the levels of genetic diversity and the patterns of gene structure for the KNH is very important for meeting the demands of future breeding programs and for formulating effective conservation strategies of the indigenous breeds.

In recent years, several studies have been undertaken to investigate the genetic characteristics of the KNH by using either DNA markers (Cho et al., 2002; Cho et al., 2000b; Cho et al., 2001; Oh and Jung, 2001) or biochemical markers (Cho et al., 1999; Cho et al., 2000a). There is however, no further information available for the KNH parentage verification.

In this study, we presented the genetic variability of the KNH using sixteen microsatellite markers. We also demonstrate the set up of the polymorphic microsatellites and show a series of methods suitable for the routine parentage testing in the KNH.

MATERIALS AND METHODS

Sample collection and DNA extraction

Genomic DNAs were prepared from whole blood samples, which were collected from 103 KNH including 19 samples used for parentage testing. Genomic DNAs from samples were extracted using a MagExtractor System MFX-2000 (Toyobo, Japan) according to the manufacturer's protocols (Tozaki et al., 2001).

PCR and microsatellite analysis

Sixteen microsatellites, AHT4,5, ASB2, HMS2,3,6,7, HTG4,6,7,10, VHL20, ASB17, CA425(UCDEQ425),

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Table 1. Characteristics of 16 microsatellite markers used in this study

Marker	Fluorescent dye	Primer sequences (5'→3')	Allele size range (bp)
AHT4	(FAM)-AACCGCCTGAGCAAGGAAGT,	GCTCCCAGAGAGTTTACCCT	138-170
AHT5	(JOE)-ACGGACACATCCCTGCCTGC,	GCAGGCTAAGGGGGCTCAGC	128-152
ASB2	(JOE)-CCACTAAGTGTCGTTTCAGAAGG,	CACAACTGAGTTCTCTGATAGG	222-256
ASB17	(NED)-GAGGGCGGTACCTTTGTACC,	ACCAGTCAGGATCTCCACCG	89-131
CA425	(NED)-AGCTGCCTCGTTAATTCA,	CTCATGTCCGCTTGTCTC	230-250
HMS2	(TAMRA)-CTTGCAGTCGAATGTGTATTAAAT,	ACGGTGGCAACTGCCAAGGAAG	218-238
HMS3	(TAMRA)-CCAACTCTTTGTCACATAACAAGA,	CCATCCTCACTTTTTCACTTTGTT	150-174
HMS6	(JOE)-GAAGCTGCCAGTATTCAACCATTG,	CTCCATCTTGTGAAGTGTAACTCA	153-171
HMS7	(FAM)-CAGGAAACTCATGTTGATACCATC,	TGTTGTTGAAACATACCTTGACTGT	167-189
HTG4	(FAM)-CTATCTCAGTCTTGATTGCAGGAC,	CTCCCTCCCTCCCTCTGTTCTC	127-141
HTG6	(JOE)-GAAGCTGCCAGTATTCAACCATTG,	CTCCATCTTGTGAAGTGTAACTCA	80-108
HTG7	(TAMRA)-CCTGAAGCAGAACATCCCTCCTTG,	ATAAAGTGTCTGGGCAGAGCTGCT	118-130
HTG10	(TAMRA)-CAATTCCCGCCCCACCCCCGGCA,	TTTTTATTCTGATCTGTCACATTT	89-117
LEX33	(HEX)-TTTAATCAAAGGATTCAGTTG,	GGGACACTTTCTTTACTTTC	201-221
TKY321	(HEX)-TGTGACTTCAAGAACAGACG,	ACAGTGCAAGTCTGTGAAAC	212-230
VHL20	(FAM)-CAAGTCCTCTTACTTGAAGACTAG,	AACTCAGGGAGAATCTTCCTCAG	89-107

Table 2. Allele frequencies of microsatellite DNA polymorphism in 103 Korean native horses

Loci	Alleles and frequencies																				
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
AHT4								0.1893	0.0437	0.0146	0.0534	0.1942	0.0049	0.1068	0.1893	0.2039					
AHT5										0.1068	0.1311	0.3010	0.0049	0.2039	0.2379	0.0146					
ASB2	0.1165	0.0340		0.0049				0.0097	0.0340	0.0097	0.2136	0.0146	0.0291	0.4029	0.0777	0.0146	0.0388				
ASB17					0.0194		0.0728	0.0194	0.0194	0.0825	0.0146		0.1165	0.2476	0.0777	0.0437	0.1311	0.1408	0.0146		
CA425							0.0049	0.0097	0.0340	0.3252	0.0097	0.0194	0.1650	0.4126	0.0194						
HMS2								0.3107	0.1214	0.1602	0.2718	0.1359									
HMS3									0.1748				0.1019	0.0146	0.2718	0.2233	0.1165	0.0874	0.0097		
HMS6										0.0094	0.0631	0.0291	0.1408	0.0340	0.5243	0.1990					
HMS7												0.6893	0.0631	0.0631	0.1214		0.0631				
HTG4											0.0680	0.3058	0.5000	0.0146	0.0146	0.0874	0.0097				
HTG6							0.0291		0.0388	0.0146				0.0049	0.7864	0.1117		0.0146			
HTG7										0.0049	0.1117		0.1990	0.4806	0.1602	0.0437					
HTG10									0.0388	0.0146	0.0146	0.0146	0.1505	0.0340	0.3204	0.0485	0.0146	0.3301	0.0097	0.0049	0.0049
LEX33										0.0485	0.2427	0.1505	0.0049	0.1311	0.1990		0.0534	0.0146	0.0874		0.0680
TKY321								0.0583	0.0194		0.0777	0.0680	0.1845		0.2330	0.0049	0.0194	0.2282	0.1068		
VHL20									0.1990	0.0146			0.2718		0.0291	0.3398	0.0146	0.1311			

LEX33 and TKY321 were used for the analysis of the Korean native horses, and the primers are given in Table 1. PCR reaction was accomplished in a total volume of 15 μl of the following mixture: 40 ng of genomic DNA, each primer, 1.25 mM of dNTPs, 2.5 μl of 10× reaction buffer, and 5 U of Taq polymerase (PE, USA). The PCR amplification procedure was as follows: one cycle was performed by an initial denaturation for 10 mins at 95°C, followed by 30 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. An extension step of 72°C for 60 minutes was added after the final cycle (Bozzini et al., 1996). Different annealing temperatures were used 56°C for the CA425 and LEX33 locus. Multiplex PCR systems were performed in a GeneAmp PCR System 9600 (Applied Biosystems). The PCR products were prepared for electrophoresis and loaded onto an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The samples run on an ABI PRISM 310 Genetic Analyzer were prepared by combining 1 μl of PCR product with 0.4 μl of GeneScan® 400 HD [ROX] Size Standard (Applied Biosystems) and 12 μl of deionized formamide. After the denaturation for 3 min at 90°C and a snap cooling, samples were injected electrokinetically (5 s, 15 kV) and electrophoresed at 15 kV in a Performance Optimized Polymer 4 (Applied Biosystems). Data were then collected using ABI PRISM 310 Collection Software application, Ver. 1.0.2 (Applied Biosystems), and GeneScan software Ver. 2.1 (Applied Biosystems) automatically analyzed the collected data and determined the allele sizes. Alleles were assigned by a Genotyper 2.0 Software system (Applied Biosystems) (Kakoi et al., 2000).

Practical validation for parentage verification

In the cases of 19 Korean native horses currently living, DNA typing was applied to validate the practical efficacy for parentage verification.

Table 3. Number of allele, observed heterozygosity (H_O), expected heterozygosity (H_E), polymorphic information content (PIC) and exclusion probability (PE) of microsatellite loci in 103 Korean native horses

Loci	No. of allele	H_O	H_E	PIC	PE
AHT4	9	0.835	0.837	0.810	0.664
AHT5	7	0.718	0.786	0.749	0.573
ASB2	13	0.660	0.771	0.742	0.581
ASB17	13	0.893	0.871	0.853	0.736
CA425	10	0.583	0.698	0.643	0.447
HMS2	5	0.796	0.774	0.734	0.553
HMS3	8	0.796	0.818	0.788	0.632
HMS6	7	0.650	0.663	0.621	0.436
HMS7	5	0.515	0.501	0.472	0.305
HTG4	7	0.670	0.647	0.588	0.394
HTG6	7	0.398	0.368	0.347	0.208
HTG7	6	0.699	0.693	0.649	0.459
HTG10	13	0.631	0.763	0.725	0.552
LEX33	10	0.621	0.848	0.826	0.690
TKY321	10	0.825	0.837	0.813	0.670
VHL20	7	0.641	0.756	0.712	0.526

Table 4. Allele diversity (the mean number alleles per locus), observed heterozygosity (H_O), expected heterozygosity (H_E), mean polymorphism information content (PIC) and total exclusion probability (PE) in Korean native horses

Sample size	Allele diversity	H_O	H_E	PIC	PE
103	8.56	0.683	0.727	0.692	0.9999

Statistical analysis

Allelic frequency, the number of alleles per locus was directly counted from the observed genotype and the probability of exclusion (PE), Hardy-Weinberg equilibrium (HWE) for genotype frequency and heterozygosity at each locus were computed using the CERVEX software (Marshall et al., 1998).

RESULTS

Microsatellite DNA typing

All of the primers amplified very well, with the exception of HTG10 that sometimes exhibited low peak heights, especially the L allele, this allele being unsuitable for automated scoring. Allele frequency and the heterozygosity at each locus are shown in Table 2. The number of alleles per locus varied from 5 to 13 with an average value of 8.56 in the KNH. The highest observed allele frequencies in each locus were AHT4 P (0.2039), AHT5 L (0.3010), ASB2 N (0.4029), ASB17 N (0.2476), CA425 N (0.4126), HMS2 H (0.3107), HMS3 O (0.2718), HMS6 O (0.5243), HMS7 L (0.6893), HTG4 M (0.5000), HTG6 O (0.7864), HTG7 N (0.4806), HTG10 R (0.3301), LEX33 K (0.2427), VHL20 P (0.3398) and TKY321 O (0.2330) in the KNH.

The observed heterozygosity (Ho), expected heterozygosity (H_E), polymorphic information content (PIC) and exclusion probability (PE) are shown in Table 3 and 4. The observed heterozygosity and the expected heterozygosity ranged 0.398-0.893 (the average value was 0.683) and 0.368-0.871 (the average value was 0.727) in the KNH, respectively.

The PIC value and exclusion probability ranged 0.347-0.853 (the average value was 0.692) and 0.208-0.736, respectively, and the total exclusion probability of 16 microsatellite loci was 0.9999. Of the 16 markers, AHT4, AHT5, ASB2, ASB17, HMS2, HMS3, HTG10, LEX33, TKY321 and VHL20 loci have a relatively high PIC value (>0.7) in the KNH.

Practical validation for parentage verification

A total of 19 Korean native horses were used to validate the practical efficacy for parentage verification. Of the 19 foals, 5 foals were disqualified by an incompatibility of 4-7 markers according to a Mendelian fashion in the present DNA typing for parentage testing (Table 5).

DISCUSSION

Horse breed registries rely on genetic testing as part of the process to ensure pedigree integrity. Equine blood typing and microsatellite DNA typing are now indispensable for the accurate recording of Thoroughbred horses worldwide (Bowling, 1996). Equine microsatellites were first characterized by Ellegren et al. (1992) and Marklund et al. (1994) who isolated sets of (CA)n repeats and demonstrated that they were highly polymorphic in horses. 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 the high levels of polymorphism, which can be

Table 5. The results parentage testing by 16 microsatellite DNA typing in 5 Korean native horses

Samples	Loci															
	AHT4	AHT5	ASB2	ASB17	CA425	LEX33	HMS2	HMS3	HMS6	HMS7	HTG4	HTG6	HTG7	HTG10	TKY321	VHL20
Sire	L/P*	L/L	K/N	N/Q	M/M	K/K	H/I	I/O	O/P	L/Q	L/M	O/O	N/O	M/R	R/S	M/P
Dam	P/P	L/O	K/N	M/N	J/N	K/N	H/I	O/Q	M/O	L/L	L/P	O/O	N/O	R/R	Q/R	P/P
Foal I	H/P	K/L	N/N	I/M	J/N	K/N	H/K	O/O	M/O	L/L	M/P	O/O	M/N	O/R	O/R	M/P
Sire	L/P	L/L	K/N	N/Q	M/M	K/K	H/I	I/O	O/P	L/Q	L/M	O/O	N/O	M/R	R/S	M/P
Dam	H/L	J/L	A/A	O/R	N/N	J/J	H/K	M/P	K/O	L/O	K/M	K/O	M/M	O/R	H/H	P/P
Foal II	H/L	J/L	A/N	M/O	M/N	N/N	H/K	M/O	K/M	L/L	K/M	K/M	M/M	O/R	H/R	M/P
Sire	L/P	L/L	K/N	N/Q	M/M	K/K	H/I	I/O	O/P	L/Q	L/M	O/O	N/O	M/R	R/S	M/P
Dam	K/L	L/L	K/Q	M/N	J/N	K/N	I/K	Q/R	O/O	L/O	M/M	O/P	N/P	R/R	O/R	P/P
Foal III	H/K	L/L	K/K	M/N	J/N	K/K	H/K	O/R	O/O	L/L	M/M	O/P	N/N	O/R	O/R	M/M
Sire	L/P	L/L	K/N	N/Q	M/M	K/K	H/I	I/O	O/P	L/Q	L/M	O/O	N/O	M/R	R/S	M/P
Dam	H/L	K/N	N/N	M/P	J/M	J/N	J/K	M/Q	M/O	L/N	M/P	O/O	K/M	N/R	O/S	O/P
Foal IV	L/L	L/N	K/N	M/Q	L/M	K/N	I/K	M/Q	O/P	L/O	M/P	G/O	K/O	M/R	R/S	P/P
Sire	L/P	L/L	K/N	N/Q	M/M	K/K	H/I	I/O	O/P	L/Q	L/M	O/O	N/O	M/R	R/S	M/P
Dam	L/L	N/N	N/N	N/N	J/N	K/K	H/L	M/Q	O/P	L/O	L/M	G/O	N/N	O/R	K/M	M/M
Foal V	P/P	L/N	K/N	G/Q	N/N	K/K	H/I	O/O	O/P	L/Q	L/L	O/O	N/O	M/R	M/R	M/P

* Allele codes are identical to the alphabetical and numerical symbols used on 2000 ISBC/ISAG Horse Comparison Test.

easily scored by a computer program. It has been shown that the DNA typing can be analyzed semi-automatically (Tozaki, 2001). ISBC has required a higher probability of exclusion (PE) value for parentage verification and individual identification in horses (Tozaki et al., 2001). The PE is a parameter used to solve the problems of some genetic markers in a population (Vegapla et al., 1998) and it is the most commonly used as a molecular marker in pedigree verification (Luikart et al., 1999). In previous studies, the total PE of the blood typing (serological and biochemical polymorphisms) in the KNH was 0.9656 (Cho et al., 2000b).

The Horse Genetic Committee of International Society for Animal Genetics (ISAG) presented 9 microsatellite markers (AHT4, AHT5, ASB2, HMS3, HMS6, HMS7, HTG4, HTG10, VHL20) as the international minimum standard for the microsatellite marker systems used in typing for horse parentage testing (Tozaki et al., 2001).

Our present objective was to construct a paternity testing system for individual identification and parentage verification of the KNH and to investigate a validation of 16 microsatellite markers for routine KNH parentage testing and polymorphisms in the population of the KNH. In this study, our estimate (H_O, H_E) of genetic diversity in the KNH (H_O=0.683, H_E=0.727) shows a lower value than that of the crossbreed horse (Thoroughbred horse×Korean native horse, CBH, H_O=0.789, H_E=0.785). However, our estimate (H_O, H_E) of genetic diversity in the KNH shows a higher level of diversity than that (H_O=0.657, H_E=0.711) of the Thoroughbred horse reported by Cho (2002). A PE value (PE=0.9999) in the KNH is also higher than that (PE=0.9979) of the Thoroughbred horse reported by Cho (2002) and that (PE=0.9980) of the KNH reported by Han et al. (2002). The differences of the genetic diversity value surveyed could probably be explained by the choice of the microsatellite markers. ISBC has demanded a higher PIC and PE value, more than 0.9995, for paternity testing of the Thoroughbred horses. Of the 19 foals selected for parentage testing by DNA typing, 5 foals (26.3%) were disqualified by incompatibility of 4-7 markers according to a Mendelian fashion in the present DNA typing. These results suggest that the present DNA typing has a practical efficacy for the KNH parentage verification. From the present study we concluded that the selection of the microsatellites was considered to be important and also, it was effective in resolving parentage testing. The PIC value of over 0.7 for each microsatellite on the average was required, and a total PE value of over 0.9995 was required when using the 10 microsatellite (AHT4, AHT5, ASB2, ASB17, HMS2, HMS3, HTG10, LEX33, TKY321, VHL20) for the KNH parentage testing. These results can give basic information for developing accurate parentage verification and individual identification system in the KNH.

REFERENCES

Bowling, A. T. 1996. Horse genetics. CAB International, pp. 82-96.

Bowling, A. T. 1997. Nine equine dinucleotide repeats at microsatellite loci UCDEQ136, UCDEQ405, UCDEQ412, UCDEQ425, UCEDQ437, UCDEQ467, UCDEQ487, UCDEQ502 and UCDEQ505. Anim. Genet. 28:370-371.

Bozzini, M., D. Fantin, J. S. Ziegle, H. Van Haeringen, W. Jacobs, M. Ketcham, M. Spencer and S. Bates. 1996. Automated equine paternity testing. Anim. Genet. 27:32.

Cho, G. J., T. S. Kim, Y. H. Um, B. H. Kim and J. S. You. 1999. Genetic studies of blood markers in Cheju horses. I. Red blood cell types. Kor. J. Vet. Res. 39(6):1066-1072.

Cho, G. J., B. H. Kim, D. S. Lee and K. K. Lee. 2000a. Genetic studies of blood markers in Cheju horses. II. Blood protein types. Kor. J. Vet. Res. 40(2):283-290.

Cho, G. J., B. H. Kim, D. S. Lee and J. A. Shin. 2000b. Usefulness of microsatellite markers for horse parentage testing. Kor. J. Genetics 22(4):281-287.

Cho, B. W., K. W. Lee, H. S. Kang, S. K. Kim, T. S. Shin and Y. G. Kim. 2001. Application of polymerse chain reaction with short

oligonucleotide primers of arbitrary sequence for the genetic analysis of Cheju native horse. J. Agri. Tech. Dev. Inst. 5:109-114.

Cho, G. J. 2002. Microsatellite DNA polymorphism of Thoroughbred horses in Korea. Kor. J. Genetics 24(2):177-182.

Dewoody, J. A. and J. C. Avise. 2000. Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. J. Fish Biology 56:461-473.

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

Han, S. K., H. D. Byun and E. R. Chung. 2002. Genetic analysis of Korean native horses using microsatellite markers(abstract only). Proc. 28th ISAG Conf. Anim. Genet. pp. 101-102.

Kim, K. S., J. S. Yeo, J. W. Lee, J. W. Kim and C. B. Choi. 2002. Genetic diversity of goats from and China using microsatellite analysis. Asian-Aust. J. Anim. Sci. 15:461-265.

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

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

Marshall, T. C., J. Slate, L. Kruuk and J. M. Pemberton. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Molecular Ecology 7:639-655.

Oh, M. Y. and Y. H. Jung. 2001. Mitochodrial DNA Polymorphism in Cheju and Tsushima Native Horses Using SSCP Analysis. Kor. J. Genetics 23(1):35-43.

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

Tozaki, T., H. Kakoi, S. Mashima, K. I. Hirota, T. Hasegawa, N. Ishida, N. Miura, N. H. Choi-Miura and M. Tomita. 2001. Population Study and Validation of Paternity Testing for Thoroughbred Horses by 15 Microsatellite Loci. J. Vet. Med. Sci. 63(11):1191-1197.

Vegapla, J. L., P. P. Rodriguezgallardo and M. J. Zamarano. 1998. Exclusion probability in Spanish Pure Breed Horse (Andalusian Horse) with microsatellites (abstract only). Anim. Genet. 29:17.