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Parentage testing of Thoroughbred horse in Korea using microsatellite DNA typing

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The present study was to construct a parentage testing system for Thoroughbred (TB) horse. A total number of 1,285 TB horse samples including 962 foals for parentage testing, 9 sires and 314 dams for individual identification were genotyped. Genomic DNA was extracted from 5 hair roots and genotyped by using 14 microsatellite markers (AHT4, AHT5, ASB2, ASB17, ASB23, CA425, HMS1, HMS3, HMS6, HMS7, HTG4, HTG10, LEX3 and VHL20). This method consisted of multiplexing PCR procedure and showed reasonable amplification of all PCR products. Genotypes were determined by genetic analyzer. The number of alleles per locus varied from 3 to 9 with a mean value of 6.36 in TB horse. The expected heterozygosity was ranged from 0.548 to 0.831 (mean 0.699), and the total exclusion probability of 14 microstellite loci was 0.9998. Of the 14 markers, ASB2, ASB17, ASB23, HMS7 and HTG10 loci have relatively high PIC value (> 0.7). Of the 962 foals, 960 foals were qualified by compatibility according to the Mendelism. These results suggest that the DNA typing method has high potential for parentage verification and individual identification of TB horses.

Key words: genotype, horse, microsatellite, parentage verification

Thoroughbred (TB) horse is a breed of light horses improved for a purpose of horse racing in the United Kingdom. The origin of TB horse was made by crossing eastern stallions (Arabian, Barb, or Turk) with mares of English native horse, and then by the artificial selection for horse racing from early 1700's. This breed, originally a crossbred, has had a closed studbook for about 200 years [20]. Korea Racing Association has played a role of the sole authority for TB horse registration in Korea since 1993 and published the Korean Stud Book in 1998. TB horse registries have verified pedigree records and resolved queries of parentage using microsatellite DNA typing [21]. In practice, horse breeders provide a horse parentage data to breeding societies, which enter the data into the registry to generate pedigrees. One of the most reliable and efficient method for pedigree construction and analysis is 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 reagents and instruments [8].

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 [15,23].

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 horses such as native horse [2]. In cattle [11], pig [14] and canine [6], 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) [5,6].

In the present study, we performed a routine DNA typing with 14 microsatellite markers including 9 international minimum standard microsatellite markers for parentage verification and individual identification of TB horse. 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 hair roots, which were collected from 1,285 horses including 962 foals. Genomic

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Table 1. Characteristics of 14 microsatellite loci used in this study

Locus	Primer sequence(5' → 3')	Allele size (bp)	References
AHT4	(FAM)-AACCGCCTGAGCAAGGAAGT GCTCCCAGAGAGTTTACCCT	138-170	Binns *et al.* (1995)
AHT5	(VIC)-ACGGACACATCCCTGCCTGC GCAGGCTAAGGGGGCTCAGC	128-152	Binns *et al.* (1995)
ASB2	(VIC)-CCACTAAGTGTCGTTTCAGAAGG CACAACTGAGTTCTCTGATAGG	222-256	Breen *et al.* (1997)
ASB17	(PET)-GAGGGCGGTACCTTTGTACC ACCAGTCAGGATCTCCACCG	89-131	Breen *et al.* (1997)
ASB23	(VIC)-GCAAGGATGAAGAGGGCAGC CTGGTGGGTTAGATGAGAAGTC	176-212	Irvin *et al.* (1998)
CA425	(PET)-AGCTGCCTCGTTAATTCA CTCATGTCCGCTTGTCTC	230-250	Eggleston-Stott *et al.* (1997)
HMS1	(PET)-CATCACTCTTCATGTCTGCTTGG TTGACATAAATGCTTATCCTATGGC	166-178	Guerin *et al.* (1994)
HMS3	(NED)-CCAACTCTTTGTCACATAACAAGA CCATCCTCACTTTTTCACTTTGTT	150-174	Guerin *et al.* (1994)
HMS6	(JOE)-GAAGCTGCCAGTATTCAACCATTG CTCCATCTTGTGAAGTGTAACTCA	153-171	Guerin *et al.* (1994)
HMS7	(FAM)-CAGGAAACTCATGTTGATACCATC TGTTGTTGAAACATACCTTGACTGT	167-189	Guerin *et al.* (1994)
HTG4	(FAM)-CTATCTCAGTCTTGATTGCAGGAC CTCCCTCCCTCCCTCTGTTCTC	127-141	Ellegren *et al.* (1992)
HTG10	(NED)-CAATTCCCGCCCCACCCCCGGCA TTTTTATTCTGATCTGTCACATTT	89-171	Marklund *et al.* (1994)
LEX3	(PET)-ACACTCTAACCAGTGCTGAGACT GAAGGAAAAAAAGGAGGAAGAC	137-160	Coogle *et al.* (1996)
VHL20	(FAM)-CAAGTCCTCTTACTTGAAGACTAG AACTCAGGGAGAATCTTCCTGAG	89-107	van Haeringen *et al.* (1994)

DNAs from samples were extracted using MagExtractor System MFX-2000 (Toyobo, Japan) according to the manufacturer's protocols [21].

Microsatellite markers

Fourteen microsatellites were selected for this study (Table 1). These microsatellite markers have been reported by the horse applied genetics committee of ISAG for individual identification and parentage verification of TB horse.

Microsatellite analysis and parentage testing

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: first 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 [3,8]. 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 [8]. In addition, sample No. 1 from ISAG 2003/2004 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 [18].

Table 2. Allele frequencies of microsatellite DNA polymorphisms in Thoroughbred horse

Locus	No. of allele	Allele* (frequency)					
AHT4	5	H(0.2105)	J(0.2241)	K(0.1732)	L(0.0004)	O(0.3918)	
AHT5	5	J(0.1393)	K(0.4911)	M(0.2136)	N(0.1233)	O(0.0327)	
ASB2	8	B(0.0307)	K(0.1304)	M(0.1331)	N(0.1366)	O(0.1163)	P(0.0117)
		Q(0.2634)	R(0.1778)				
ASB17	9	G(0.3261)	H(0.0016)	M(0.0315)	N(0.2533)	O(0.2171)	P(0.0008)
		Q(0.0008)	R(0.1685)	S(0.0004)			
ASB23	6	I(0.0704)	J(0.2817)	K(0.2424)	L(0.1782)	S(0.2058)	U(0.0214)
CA425	7	I(0.0323)	J(0.1786)	K(0.0019)	L(0.0132)	M(0.0268)	N(0.6342)
		O(0.1128)					
HMS1	3	I(0.1739)	J(0.4553)	M(0.3708)			
HMS3	7	I(0.5475)	K(0.0004)	M(0.1451)	N(0.0393)	O(0.1089)	P(0.1467)
		R(0.0121)					
HMS6	6	K(0.1323)	L(0.0276)	M(0.2926)	O(0.0140)	P(0.5319)	R(0.0016)
HMS7	6	J(0.0864)	K(0.0012)	L(0.1412)	M(0.2385)	N(0.2023)	O(0.3304)
HTG4	5	K(0.5350)	L(0.0012)	M(0.4058)	N(0.0249)	P(0.0331)	
HTG10	8	I(0.2782)	K(0.1245)	L(0.1669)	M(0.1833)	O(0.0934)	Q(0.0008)
		R(0.1518)	S(0.0012)				
LEX3	8	F(0.0039)	H(0.2665)	J(0.0004)	L(0.0198)	M(0.1362)	N(0.1171)
		O(0.0529)	P(0.4031)				
VHL20	5	I(0.2805)	L(0.2307)	M(0.3463)	N(0.1374)	O(0.0051)	

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

Results

Heterozygosities and the number of alleles

Microsatellites were highly polymorphic in TB horse (Table 2 and 3). The number of alleles varied from 3 (HMS1) to 9 (ASB17), and the average number of alleles was 6.36 in this study. The observed heterozygosity and expected heterozygosity ranged from 0.553 to 0.852 (mean 0.663), from 0.548 to 0.831 (mean 0.699), respectively. PIC value ranged from 0.451 to 0.809 with a mean value 0.652. The total PE value of 14 microsatellite loci was 0.9998 in TB horse. These are higher than the value (0.9995) of the International Stud Book Committee (ISBC) proposition, and the 14 microsatellite markers system is theoretically considered

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

Locus	No. of allele	OHet	EHet	PIC	PE*
AHT4	5	0.781	0.722	0.674	0.474
AHT5	5	0.685	0.678	0.634	0.443
ASB2	8	0.852	0.831	0.809	0.663
ASB17	9	0.762	0.753	0.710	0.520
ASB23	6	0.779	0.783	0.748	0.571
CA425	7	0.553	0.551	0.513	0.332
HMS1	3	0.626	0.625	0.547	0.334
HMS3	7	0.645	0.644	0.608	0.424
HMS6	6	0.618	0.613	0.551	0.352
HMS7	6	0.767	0.766	0.728	0.545
HTG4	5	0.559	0.548	0.451	0.257
HTG10	8	0.804	0.814	0.788	0.630
LEX3	8	0.756	0.731	0.691	0.505
VHL20	5	0.736	0.730	0.680	0.479
Mean	6.36	0.663	0.699	0.652	0.9998**

*OHet: Observed heterozygosity, EHet: Expected heterozygosity, PIC: Polymorphic information contents, PE: Exclusion probability.
**Total exclusion probability.

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

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

to be greatly useful for parentage verification on TB horse in Korea.

Parentage verification

The results of DNA typing for parentage testing in the 962 foals are shown in Table 4. Of the 962 foals, 960 foals (99.80%) were qualified by the compatibility of 14 microsatellite markers according to Mendelian fashion in the present DNA typing for parentage verification.

Discussion

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 [19]. Also, application of the DNA markers reveal extensive capability to distinguish among individual, and this ability has been utilized in analyses of reproductive success, kinship and parentage. The aim of the present study was to construct a correct pedigree of TB horse family. After genotyping, parentage testing was performed according to Mendelism and ISAG guideline.

Equine microsatellites were first characterized by Ellegren *et al*. [10] and Marklund *et al.* [17] 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 [20].

ISBC has required a higher probability of exclusion (PE) value for parentage verification and an individual identification in horse [21]. PE is a parameter to solve problems of some genetic markers in a population and is most commonly used as molecular markers in pedigree verification [16].

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, 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 [1].

As demonstrated in this study, 960 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 7 markers. Our 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 on TB horse. These are higher than the value (0.9995) of the ISBC proposition [21]. In conclusions, the 14 microsatellite markers system is theoretically considered to be greatly useful for parentage verification on TB horse in Korea.

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