Sex Determination and Parentage Testing in Miniature Horses

Gil-jae Cho* and Byung-wook Cho[1]

Laboratory of Equine Genetics, Korea Racing Association, Gwachon 427-711, Korea, [1]Department of Animal Science, Miryang National University, Miryang 627-702, Korea

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The aim of this study was to construct a correct pedigree of miniature horses (MH). The sex of MH was detected by PCR amplification of the sex determining region of the Y chromosome gene (SRY) prior to parentage testing. Ten random MH samples for parentage testing were genotyped by using 16 microsatellite markers. Since the SRY band (430 bp) was detected in horses No. 1, 2, 6, 7, 8, 9, 10, these are male. However, the DNA segment was not identified in horses No. 3, 4, and 5, which therefore are female. After genotyping, parentage testing was performed according to Mendelian fashion and International Society for Animal Genetics (ISAG) guideline. Of the 10 MH, 3 were qualified by the compatibility of 16 markers according to Mendelian fashion in the present DNA typing for parentage verification. These results can provide basic information for developing parentage verification and an individual identification system in MH.

Key words – Microsatellite, miniature horse, parentage verification, SRY.

Miniature horses (MH) have been bred for centuries as novel pets and companion animals. The MH is not a pony because it does not possess pony-like characteristics, but is more horse-like in its body proportions and character. A good example of the modern MH is the Falabella, which was produced by first crossing very small Shetland ponies with small Thoroughbreds, and then selectively inbreeding the smallest of the offspring[11].

Studies of mammalian sexual differentiation suggest that the primary male sex determining signal is a DNA binding protein encoded by a gene on the Y chromosome referred to as the sex determining region on the Y chromosome (SRY)[8]. The equine genomic SRY gene does not contain any introns in its amino acid coding sequence similar to other mammalian species. Although specific amplification of the SRY gene was very effective for detecting the SRY gene from male genomic DNA, karyotyping is indispensable for diagnosing an infertile mare as an XY mare[8]. In XY females, lack of either SRY gene or its function may cause inappropriate development of gonads[12].

The development of molecular DNA markers has brought about great advances during recent years because of their highly polymorphic nature. Application of the DNA markers reveal extensive capability to distinguish among individuals, and this ability has been utilized in analyses of reproductive success, kinship and parentage. The term microsatellites refers to a class of codominant DNA markers which are inherited in a Mendelian fashion. Microsatellite loci are widely dispersed along and among chromosomes. Each locus is characterized by a known DNA sequence, and is typically composed of two to four nucleotides such as $(CA)_n$ or $(GATA)_n$, which n indicates from 5 to 50. Many kinds of microsatellites are informative due to their high polymorphisms and they are useful in paternity testing of horses. In cattle, pigs, horses and dogs, pedigree control has been performed on a routine basis in most countries relying on DNA typing that has been standardized through regular comparison tests under the auspices of the International Society for Animal Genetics (ISAG)[3,4,5]. There are 10 MH of unknown pedigree in Seoul Grand Park. Therefore, accurate determination of the degree of relatedness and efficient control of pedigree registration is of great importance in MH breeding. In this study, we present the genetic variability and correct pedigree of the MH using sixteen microsatellite markers.

Materials and Methods

Sample collection and DNA extraction

Genomic DNAs were extracted from hair roots, which were collected from 10 horses, using MagExtractor System MFX-2000 (Toyobo, Japan) according to the manufacturer's protocols[13].

PCR and Sex determination

Sex was analyzed by the results of PCR amplification of SRY prior to parentage testing, and the primers are given in Table 1. PCR was performed in a total volume of 50 µl of the

***Corresponding author**
Tel : +82-2-509-1933, Fax : +82-2-509-2672
E-mail : chogj@kra.co.kr

Table 1. Nucleotide sequences of primers for SRY used in this study

Primers	Primer sequences (5′→3′)
SRY forward	5′-CTTAAGCTTCTGCTATGTCCAGAGTATCC-3′
SRY reverse	5′-GCGGTTTGTCACTTTTCTGTGGCATCTT-3′

following mixture: 30 ng of genomic DNA, 30 pmols each of 2 primers, 12.5 µl premix reaction buffer (ABgene, USA). PCR amplification was as follows: first step was performed by initial denaturation for 5 min at 94℃, followed by 35 cycles at 94℃ for 60 sec and 68℃ for 30 sec. PCR products were analyzed by 4% agarose gel electrophoresis of SRY gene fragments[8].

Microsatellite analysis and Parentage testing

Sixteen microsatellites, AHT4, AHT5, ASB2, ASB17, ASB23, CA425, HMS1, HMS3, HMS6, HMS7, HTG4, HTG10, LEX3, LEX33, TKY321 and VHL20 were selected for this study[3], and the primers are given in Table 2. These microsatellite markers have been reported by the horse applied genetics committee of ISAG for individual identification and parentage verification of Thoroughbreds.

Microsatellite markers were combined in multiplex PCR using fluorescently labelled primers and amplified in a total volume of 15 µl of the following mixture: each primer in 40 ng of genomic DNA, 1.25 mM of dNTPs, 2.5 µl of 10x 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℃, followed by 30 cycles at 95℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 1 min. An extension step of 72℃ for 60 min was added after the final cycle[2]. However, the annealing temperature was perfomed at 56℃ for LEX33 and TKY321 locus. 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-ROX 400 (Applied Biosystems, USA) was used for sizing alleles. In addition, sample No. 1 from the International Society for Animal Genetics (ISAG) 2001/2002 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.

Results

Sex determination

PCR products of SRY following agarose gel electrophoresis are presented in Fig. 1. Since the SRY band (430 bp) was detected in horses No. 1, 2, 6, 7, 8, 9, 10, these are male. However, the DNA segment was not identified in horses No. 3, 4, and 5, which therefore are female.

Parentage verification

The results of DNA typing for parentage testing in the 10 MH are shown in Table 3. Of the 10 MH, 3 were qualified by the compatibility of 16 markers according to Mendelian fashion in the present DNA typing for parentage verification.

Table 2. Characteristics of 16 microsatellite markers used in this study

Marker	Primer sequences (5′ → 3′)		Range of allele size (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
ASB23	(VIC)- GAGGTTTGTAATTGGAATG	GAGAAGTCATTTTTAACACCT	176-212
CA425	(NED)-AGCTGCCTCGTTAATTCA	CTCATGTCCGCTTGTCTC	230-250
LEX3	(PET)- ACATCTAACCAGTGCTGAGACT	GAAGGAAAAAAAGGAGGAAGAC	137-160
LEX33	(HEX)-TTTAATCAAAGGATTCAGTTG	GGGACACTTTCTTTACTTTC	201-221
HMS1	(PET)- CATCACTCTTCATGTCTGCTTGG	TTGACATAAATGCTTATCCTATGGC	166-178
HMS3	(TAM)-CCAACTCTTTGTCACATAACAAGA	CCATCCTCACTTTTTCACTTTGTT	150-174
HMS6	(JOE)-GAAGCTGCCAGTATTCAACCATTG	CTCCATCTTGTGAAGTGTAACTCA	153-171
HMS7	(FAM)-CAGGAAACTCATGTTGATACCATC	TGTTGTTGAAACATACCTTGACTGT	167-189
HTG4	(FAM)-CTATCTCAGTCTTGATTGCAGGAC	CTCCCTCCCTCCCTCTGTTCTC	127-141
HTG10	(TAM)-CAATTCCCGCCCCACCCCCGGCA	TTTTTATTCTGATCTGTCACATTT	89-117
TKY321	(HEX)-TGTGACTTCAAGAACAGACG	ACAGTGCAAGTCTGTGAAAC	212-230
VHL20	(FAM)-CAAGTCCTCTTACTTGAAGACTAG	AACTCAGGGAGAATCTTCCTCAG	89-107

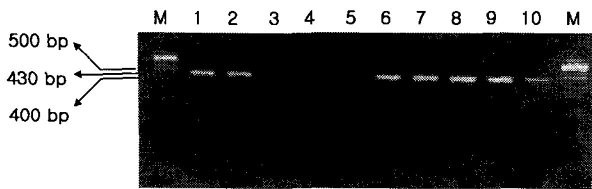

Fig. 1. Agarose gel electrophoresis of SRY gene fragments. Lane M: 100-bp DNA ladder; SRY band of 430 bp DNA segment was obtained by PCR on lane 1-2, 6-10, but wasn't lane 3-5.

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. The aim of the present study was to construct a correct pedigree of a MH family, which could not been determined by blood group systems. Prior to microsatellite typing, the sex of the MH was detected by PCR amplification of SRY gene. After genotyping, paternity testing was performed according to Mendelian fashion and ISAG guideline.

Equine microsatellites were first characterized by Ellegren *et al*[7] and Marklund *et al*[10] who isolated a set 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 their high levels of polymorphism, which can be easily scored by a computer program. This indicates that DNA typing can be analyzed semi-automatically[13]. The International Stud Book Committee (ISBC) has required a high probability of exclusion (PE) value for parentage verification and an individual identification in horses[14]. PE is a parameter to solve problems of some genetic markers in a population[15] and is most commonly used as molecular markers in pedigree verification[9]. Microsatellites used for equine parentage testing in this study showed greater variability than blood typing systems. All alleles of the microsatellites were correctly inherited to the next generation[13].

The Horse Genetic Committee of the International Society for Animal Genetics (ISAG) presented 9 microsatellite markers (AHT4, AHT5, ASB2, HMS3, HMS6, HMS7, HTG4, HTG10, VHL20) as international minimum standard microsatellite marker systems, as well as additional markers (ASB17, ASB23, CA425, HMS1, LEX3, LEX33, TKY321) to be typed for horse parentage testing[14]. 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.

As demonstrated in this study, SRY gene is a powerful tool for horse sex determination, and 3 MH (No. 7, 9, 10) inherited alleles from sire and dam, and were qualified by the compatibility of 16 markers according to Mendelian fashion in the present DNA typing. Thus, the three MH, No. 7, 9 and 10, were qualified as the foals of horses No. 2 and 3, No. 8 and 4, and No. 4, respectively.

These results are enable to perform a quick diagnosis for sex determination prior to cytogenetic analysis, and can provide basic information for developing parentage verification and an individual identification system in MH.

Table 3. Parentage testing using 16 microsatellite markers in miniature horses

Sample No.	Loci																Remarks
	AHT4	AHT5	ASB2	ASB17	ASB23	CA425	HMS1	HMS3	HMS6	HMS7	HTG4	HTG10	LEX3	LEX33	TKY321	VHL20	
2	J/N*	N/N	N/Q	N/N	I/S	M/O	J/N	M/M	K/K	N/N	M/P	I/K	N/-	K/L	I/Q	I/I	
3	K/O	N/N	M/M	F/N	K/S	G/L	J/M	M/P	O/P	N/O	L/M	O/R	K/M	K/L	H/S	M/P	P.V**
7	J/O	N/N	M/Q	F/N	K/S	G/M	J/J	M/M	K/O	N/O	L/M	I/O	M/-	K/K	H/I	I/P	
8	I/K	J/N	M/R	N/N	K/S	G/K	M/N	M/M	O/P	O/O	M/P	L/L	M/-	K/L	Q/Q	M/P	
4	J/N	J/N	M/N	K/K	I/J	F/N	M/M	R/R	P/P	L/N	M/M	I/L	K/K	K/L	M/O	J/M	P.V
10	I/N	J/N	M/R	K/N	J/K	K/N	M/N	M/R	P/P	N/O	M/P	I/L	K/-	L/L	O/Q	M/M	
4	J/N	J/N	M/N	K/K	I/J	F/N	M/M	R/R	P/P	L/N	M/M	I/L	K/K	K/L	M/O	J/M	P.V
9	J/N	J/K	N/N	K/N	I/I	F/M	J/M	M/R	K/P	N/N	M/M	I/R	K/-	K/L	I/M	I/M	
1	J/K	N/O	N/Q	Q/Q	J/S	N/N	M/M	I/R	P/P	L/M	M/M	I/L	K/-	L/O	K/M	J/J	
5	J/N	J/N	N/Q	N/N	J/S	O/O	J/M	O/R	K/O	L/M	M/M	I/L	N/N	K/P	I/S	I/N	I.I
6	I/K	N/N	M/R	F/N	S/S	G/G	N/N	M/M	K/O	O/O	P/P	L/O	N/-	K/L	H/Q	P/P	

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

**P.V : parentage verification, I.I: individual identification.

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초록 : Miniature 말의 성(sex) 결정과 친자감정

조길재* · 조병욱[1]

(한국마사회 유전자검사실, [1]밀양대학교 동물자원학과)

서울대공원에서 사육중인 Miniature horse 10두의 혈통정립을 목적으로 PCR에 의한 성별 판정 및 16개 microsatellite marker를 사용하여 친자감정을 실시하였다. 성별 판정에서 430 bp의 SRY band가 관찰된 7두는 숫말로 판정되었고, 친자감정에서는 멘델의 유전양식에 부합된 3두가 친자관계가 확인되었다. 향후 Miniature horse의 혈통보존 및 관리에 유용한 자료가 될 것으로 사료된다.