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Standardization and Usefulness of ISAG Microsatellite Markers for Individual Identification and Parentage Verification in Horse Breeds

Do-Yeon Kwon and Gil-Jae Cho*[1]

College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Korea
**Research Institute of Horse Industry, Kyungpook National University, Sangju 742-711, Korea*

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Abstract : The present study demonstrates a new approach that enables effective horse parentage testing using 22 ISAG microsatellite markers involving 6 heads of Thoroughbred horse (TB) and non-TB. In the comparison allele distribution between these horses, the alleles found in the TB were numerously detected in the non-TB. As results, we confirmed that these ISAG microsatellite markers might apply the pedigree registration of Korean native horse (Jeju horse).

Key words **:** horse, ISAG microsatellite marker, allele, pedigree registration.

Introduction

Horse breeds are improved for various purposes, and more than 200 breeds are distributed in the world. These breeds have peculiar shapes and characteristics, which have been based on pure breed management. Since individual identification and parentage verification can be a basic method for scientific and pure breed control, many studies for horse parentage verification have been performed (1,3,4,5,7,18,26).

DNA-based genetic testing is used for most domesticated animals to confirm identity, to determine parentage and, particularly, to validate registries (2,6,9,12,15,16,22,23).

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 (17,20,29). 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. Due to this reason, microsatellites analysis is useful for parentage testing and individual identification in forensic sciences (5,15).

Standardized genetic tests are important for sharing information, combining datasets and assisting with population management. These tests are particularly important for purebreds, especially when individuals transfer between registries and countries. The scientific community provides oversight of industry standards pertaining to parentage and identification panels (19).

In cattle (12), pig (22,23) and canine (15), 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)(4). At 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 (9).

Korea Racing Authority and Jeju Special Self-Governing Province have played a role the sole authority for Thoroughbred horse (TB) and Jeju horse (Korean native horse) registration in Korea, respectively. These registries of horse have verified pedigree records and resolved queries of parentage using microsatellite DNA typing. However, the microsatellite markers are differented with ISAG markers in Jeju horse. This study was conducted to standardize the application of ISAG horse markers for parentage verification and individual identification in horse breeds, especially Jeju horse.

Materials and Methods

Animals and Microsatellites

Genomic DNAs from 20 horse blood samples (1 Cob horse, 1 Connemara pony, 8 Irish Draught horse, 6 TB, 3 Thoroughbred and Irish Draught Cross horse, 1 Tobiano horse) for 2007/2008 ISAG horse comparison test were extracted by QIAamp DNA Blood Maxi Kit according to the manufacturer's protocols. Twenty-two ISAG microsatellite markers 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 horse breeds.

PCR, Genotyping and Parentage testing

Microsatellite markers were combined in multiplex PCR

[1]Corresponding author.
E-mail : chogj@knu.ac.kr

Table 1. The primer sequences of the microsatellite loci used in this study

Loci	Primer sequence (5'→3')	Reference
AHT4	(FAM)-AACCGCCTGAGCAAGGAAGT, GCTCCCAGAGAGTTTACCCT	Binns et al.(1)
AHT5	(JOE)-ACGGACACATCCCTGCCTGC, GCAGGCTAAGGGGGCTCAGC	Binns et al.(1)
ASB2	(JOE)-CCACTAAGTGTCGTTTCAGAAGG, CACAACTGAGTTCTCTGATAGG	Breen et al.(2)
ASB17	(NED)-GAGGGCGGTACCTTTGTACC, ACCAGTCAGGATCTCCACCG	Breen et al.(2)
ASB23	(VIC)-GCAAGGATGAAGAGGGCAGC, CTGGTGGGTTAGATGAGAAGTC	Irvin et al.(14)
CA425	(NED)-AGCTGCCTCGTTAATTCA, CTCATGTCCGCTTGTCTC	Eggleston-Stott et al.(10)
HMS1	(PET)-CATCACTCTTCATGTCTGCTTGG, TTGACATAAATGCTTATCCTATGGC	Guerin et al.(13)
HMS2	(TAM)-CTTGCAGTCGAATGTGTATTAAAT, ACGGTGGCAACTGCCAAGGAAG	Guerin et al.(13)
HMS3	(TAM)-CCAACTCTTTGTCACATAACAAGA, CCATCCTCACTTTTTCACTTTGTT	Guerin et al.(13)
HMS6	(JOE)-GAAGCTGCCAGTATTCAACCATTG, CTCCATCTTGTGAAGTGTAACTCA	Guerin et al.(13)
HMS7	(FAM)-CAGGAAACTCATGTTGATACCATC, TGTTGTTGAAACATACCTTGACTGT	Guerin et al.(13)
HTG4	(FAM)-CTATCTCAGTCTTGATTGCAGGAC, CTCCCTCCCTCCCTCTGTTCTC	Ellegren et al.(11)
HTG6	(JOE)-GAAGCTGCCAGTATTCAACCATTG, CTCCATCTTGTGAAGTGTAACTCA	Ellegren et al.(11)
HTG7	(TAM)-CCTGAAGCAGAACATCCCTCCTTG, ATAAAGTGTCTGGGCAGAGCTGCT	Marklund et al.(21)
HTG10	(TAM)-CAATTCCCGCCCCACCCCCGGCA, TTTTTATTCTGATCTGTCACATTT	Marklund et al.(21)
LEX3	(PET)-ACACTCTAACCAGTGCTGAGACT, GAAGGAAAAAAAGGAGGAAGAC	Coogle et al.(8)
LEX33	(HEX)-TTTAATCAAAGGATTCAGTTG, GGGACACTTTCTTTACTTTC	Coogle et al.(8)
TKY279	(FAM)-GCCACTCCGGTAACAAAATC, AATGAATGAGACTTGAACCC	Tozaki et al.(25)
TKY287	(FAM) -ATCAGAGAACACCAAGAAGG, TCTCTGCTATAGGTAAGGTC	Tozaki et al.(25)
TKY297	(HEX)-GTCTTTTTGTGCCTCTGGTG, TCAGGGGACAGTGGCAGCAG	Tozaki et al.(27)
TKY321	(HEX)-TAGTGTATCCGTCAGAGTTCAAAG, GCAAGGAAGTCAGACTCCTGGA	Guerin et al.(13)
VHL20	(FAM)-CAAGTCCTCTTACTTGAAGACTAG, AACTCAGGGAGAATCTTCCTCAG	Van Haeringen et al.(28)

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 10x reactionbuffer, 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 (9). Annealing temperature made different at 56°C for LEX33, TKY279, TKY287, TKY297 and TKY321. 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 3130xl Genetic Analyzer (Applied Biosystems, USA) using the recommended protocols. Size analyses of DNA fragments separated were performed with GeneMapper 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. Allelic nomenclature was in alphabetical order by size, from smallest to largest in two base pair increments. The number of alleles per locus was estimated by direct counting from observed genotype.

After genotyping, parentage testing with TB and Cross-breed foals was performed according to Mendelian fashion and ISAG guideline.

Results

Number of allele and Allele distribution

The results of analysis for two horse breed groups, TB and non-TB are shown in Table 2. The number of alleles varied from 4 (HTG4 and HTG7) to 8 (ASB2, ASB17, HTG10, LEX33 and TKY297) in the two horse breed groups. A total number of 145 (mean 6.59), 80 and 143 alleles were detected across the 22 loci analyzed in the TB and non-TB, respectively. All microsatellite loci were polymorphic in this study. In the comparison of allele distribution between these horses, the alleles found in the TB were numerously detected in the non-TB.

Parentage testing

The results of parentage testing in 2 foals (TB foal, TB and Irish Draught Cross breed, respectively) are shown in

Table 2. Number of allele and allele distribution of microsatellite DNA polymorphisms in 20 horse breeds

Loci	Allele of TB	Allele of non-TB	Allele of all horse breeds	No. of allele
AHT4	H, J, O	H, I, J, K, L, O, P	H, I, J, K, L, O, P	7
AHT5	J, K, M, N, O	J, K, L, M, N, O	J, K, L, M, N, O	6
ASB2	B, K, N, O, Q, R	I, K, M, N, O, Q, R	B, I, K, M, N, O, Q, R	8
ASB17	G, N, O	F, G, K, M, N, O, R, S	F, G, K, M, N, O, R, S	8
ASB23	I, J, K, S	I, J, K, L, S, Q, U	I, J, K, L, S, Q, U	7
CA425	J, N	F, G, I, J, M, N, O	F, G, I, J, M, N, O	7
HMS1	I, J, M	I, J, K, M, N	I, J, K, M, N	5
HMS2	J, K, L	H, J, K, L, M, R	H, J, K, L, M, R	6
HMS3	I, M, O, P	I, M, N, P, Q, R	I, M, N, O, P, Q, R	7
HMS6	M, P	K, L, M, N, O, P	K, L, M, N, O, P	6
HMS7	J, L, M, O	J, L, M, N, O, Q	J, L, M, N, O, Q	6
HTG4	K, M	K, L, M, O	K, L, M, O	4
HTG6	G, I, J	G, I, J, M, O, R	G, I, J, M, O, R	6
HTG7	K, N, O	K, M, N, O	K, M, N, O	4
HTG10	I, K, L, M, O	I, K, L, M, N, O, P, R	I, K, L, M, N, O, P, R	8
LEX3	H, L, M, P	F, H, L, M, N, O, P	F, H, L, M, N, O, P	7
LEX33	L, M, Q	K, L, M, N, O, P, Q, R	K, L, M, N, O, P, Q, R	8
TKY279	J, M, N, O	J, M, N, O, P, Q	J, M, N, O, P, Q	6
TKY287	K, N, R	K, N, O, P, Q, R, S	K, N, O, P, Q, R, S	7
TKY297	L, M, N, O	J, L, M, N, O, P, R, S	J, L, M, N, O, P, R, S	8
TKY321	I, L, M, R, S	H, I, L, M, O, R, S	H, I, L, M, O, R, S	7
VHL20	I, L, M, N, O	I, L, M, N, O, P, R	I, L, M, N, O, P, R	7
Total	80	143	145	145

*Alphabetical allele codes for all loci are identical to the assignment on 2005/2006 ISAG horse comparison test.

Table 3. 2 foals were not inherited alleles from sire or dam, and excluded by the incompatibility of 13 (AHT5, ASB2, ASB23, HMS1, HMS2, HMS3, HTG10, LEX3, TKY279, TKY287, TKY297 and VHL20 in TB) and 17 (AHT4, AHT5, ASB2, ASB17, ASB23, CA425, HMS1, HMS2, HMS3, HMS7, HTG4, HTG10, LEX3, LEX33, TKY279, TKY287 and TKY297) markers, respectively.

Discussion

Equine microsatellites were first characterized by Ellegren et al. (11) and Marklund et al. (21) 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.

The aim of the comparison test of ISAG is to enable laboratories working on immunogenetics and to maintain high standards in DNA and biochemical polymorphisms of animals. Furthermore, it is valuable to entered into international agreement on nomenclature and typing procedures. Participants in comparison test organized on behalf of ISAG must be institutional members of the society and must abide by these test guideline. Only members who paid their dues could participate. Each laboratory should be given a registered laboratory code (for example, Laboratory of Equine Science, Kyungpook National University, KOR/D). According to International Agreement on Breeding and Racing (IABR), the laboratory have to ensure that the individual identification and parentage verification for registration are only undertaken by a laboratory appointed by that Stud Book Authority, and that the laboratory is affiliated to the ISAG and participates in the comparison test organized by that society. Also, International Stud Book Committee (ISBC) and the Horse Applied Genetic Committee of ISAG have required a higher accuracy value more than 98% for parentage verification and individual identification in TB.

At present, the Horse Applied Genetic Committee of ISAG presented 9 microsatellite markers (AHT4, AHT5, ASB2, HMS3, HMS6, HMS7, HTG4, HTG10 and VHL20) as international minimum standard microsatellite marker, but will be changed 12 microsatellite loci for horse parentage testing and individual identification from next year. The Committee has recommended that parentage testing should consist of an

Table 3. Two cases of parentage testing by 22 microsatellite loci in two horse breeds

Samples		Breeds	Loci											
			AHT 4	AHT 5	ASB 2	ASB 17	ASB 23	CA 425	HMS 1	HMS 2	HMS 3	HMS 6	HMS 7	HTG 4
Case 1	Sire	TB	J/O	N/O	B/Q	G/G	I/S	J/N	M/M	L/L	I/O	M/P	L/M	K/K
	Dam	TB	J/O	K/K	K/O	N/O	J/K	N/N	I/M	J/L	I/O	M/P	M/M	K/M
	Foal	TB	O/O	J/M	K/N	G/N	L/S	J/N	J/J	K/L	I/M	M/M	M/M	K/K
Case 2	Sire	TB	H/J	N/O	B/R	N/O	I/S	N/N	I/M	L/L	I/P	M/P	L/M	K/K
	Dam	ID	K/L	J/M	M/N	M/O	I/L	J/N	J/M	K/L	I/P	M/O	M/O	K/K
	Foal	CB	J/O	N/O	K/Q	O/R	U/U	J/M	I/K	H/L	N/O	M/P	J/N	K/L

*TB: Thoroughbred horse, ID: Irish Draught horse, CB: Thoroughbred and Irish Draught Cross horse.

Table 3. continued

Samples		Breeds	Loci									
			HTG 6	HTG 7	HTG 10	LEX 3	LEX 33	TKY 279	TKY 287	TKY 297	TKY 321	VHL 20
Case 1	Sire	TB	G/O	N/N	K/M	H/H	M/M	O/O	R/R	M/O	I/L	N/N
	Dam	TB	G/J	K/O	I/M	H/L	M/Q	M/O	N/R	O/O	I/L	M/N
	Foal	TB	G/J	N/O	K/O	M/M	M/Q	J/N	K/R	L/M	M/R	I/N
Case 2	Sire	TB	J/O	N/O	K/O	M/M	L/L	N/N	K/R	L/N	I/M	I/L
	Dam	ID	J/O	K/O	K/K	N/N	K/P	M/O	K/K	L/R	I/S	L/O
	Foal	CB	J/J	N/O	L/O	H/H	K/P	O/P	N/R	L/M	I/M	I/L

*TB: Thoroughbred horse, ID: Irish Draught horse, CB: Thoroughbred and Irish Draught Cross horse

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 test for additional markers or mutation analysis (1).

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 (24). One of the most important aspects of the DNA marker panel for parentage applications is the correct exclusion of non fathers. The ability to resolve paternity when closely related individuals are tested as alleged fathers is particularly critical in inbred populations. Most microsatellites tested for the panel had comparable variation over all breeds, so the selection of microsatellites was based on other standard criteria, such as small product size, robustness of amplification and clarity in scoring. Markers panels developed for forensic purposes ultimately need to be concerned with efficiency for amplifying trace amounts of DNA and degraded DNA (19).

The present study demonstrates a new approach that enables effective horse parentage testing using 22 ISAG microsatellite markers, and apply the pedigree registration for conservation of Korean native horse. All microsatellite loci were polymorphic in this study; however, the detection of allele is also important in genotyping. Allele sizes varied among instruments, specification of size range, and levels of expertise and experience. The use of standard DNA controls allows for proper conversion and data sharing. In the comparison allele distribution between these horses, the alleles found in the TB were numerously detected in the non-TB. This suggests that the judgment criterion for registration by evaluating allele distribution needs to be revised, especially for basic registration in the Jeju horse. Also, after genotyping, parentage testing was performed according to Mendelian fashion and ISAG guideline. As demonstrated in this study, 2 foals were not inherited alleles from sire or dam, and excluded by the incompatibility of 13 and 17 markers, respectively. From our data, we confirmed that these ISAG microsatellite markers might apply the pedigree registration of Korean native horse and useful tools for managing to breed Jeju horse. This suggests that the present ISAG microsatellite markers will be also valuable markers for parentage testing and individual identification of horse breeds in Korea.

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말에서 개체식별 및 친자확인을 위한 ISAG Microsatellite Marker의 유용성 및 표준화

권도연 · 조길재*[1]

경북대학교 수의과대학, *경북대학교 말산업연구원

요 약 : 말에서 개체식별 및 친자확인을 위한 ISAG microsatellite marker의 적용을 위한 유용성 및 표준화를 위해 더러브렛종 6두를 포함한 다양한 품종의 20두를 대상으로 22개의 ISAG microsatellite marker를 분석한 결과 대립유전자의 수는 4개에서 8개로 분포하였고 더러브렛종에서 출현하는 대립유전자의 대부분이 비더러브렛종에서도 출현하였다. 본 연구결과를 통해서 ISAG microsatellte marker를 제주말의 혈통등록에 적용할 수 있음을 알 수 있었다.

주요어 : 말, ISAG Microsatellite marker, 대립유전자, 혈통등록