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Population Study and Validation of Paternity Testing for Thoroughbred Horses by 15 Microsatellite Loci

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ABSTRACT. Microsatellite 15 TKY System was characterized for parentage verification of horse registry. The Microsatellite 15 TKY System was constructed by using 15 microsatellites, *TKY279*, *TKY287*, *TKY294*, *TKY297*, *TKY301*, *TKY312*, *TKY321*, *TKY325*, *TKY333*, *TKY337*, *TKY341*, *TKY343*, *TKY344*, *TKY374*, and *TKY394*, to provide stringent PCR-based microsatellite typing specifically optimized for multicolor fluorescence detection. The Microsatellite 15 TKY System showed good resolutions for 250 unrelated Thoroughbred horses, and the probability of exclusion (PE) at each microsatellite ranged from 0.437 to 0.621, resulting in a total PE value of 99.998% for Thoroughbred horses. These results indicated that the Microsatellite 15 TKY System is useful for paternity testing of Thoroughbred horses. A paternity testing case for a Thoroughbred horse family, in which candidate sires had close relations, was analyzed using the Microsatellite 15 TKY System. In this case, the Microsatellite 15 TKY System excluded paternity of a false sire. We concluded that the Microsatellite 15 TKY System can give sufficient and reliable information for paternity testing.

KEY WORDS: microsatellite, Microsatellite 15 TKY System, paternity testing, PCR, Thoroughbred.

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Horse breed registries worldwide rely on genetic testing to protect studbook integrity. At present, paternity testing of horses is carried out using serological and biochemical polymorphisms (i.e. blood group systems EAA, EAC, EAD, EAK, EAP, EAQ and EAU and protein polymorphic systems ALB, A1B, ES, GC, HBA, PGD, PI and TF) [1, 2, 5–7, 9, 12, 15, 18, 21, 22]. Total probability of exclusion (PE) of the system is 90–97% [3]. Recently, International Stud Book Committee (ISBC) has required a higher PE value. Therefore, although it is necessary to increase the number of markers, which show polymorphisms by agglutination or gel electrophoresis, for paternity testing that has the higher PE value, it is difficult to identify novel polymorphic blood group-proteins.

Microsatellites are highly polymorphic and abundant sequences dispersed throughout most eukaryotic nuclear genomes [13, 31]. Microsatellites have the following characteristics: (i) they have a simple and stable inheritance when they are transmitted from one generation to the next; (ii) due to their small size, they are efficiently amplified using PCR techniques and can be easily resolved using polyacrylamide gel or capillary electrophoresis. Accordingly, microsatellites have been used for parentage testing in forensic sciences [8, 19, 24].

The well-known abundant microsatellites reported in the equine genome are (CA)n repeats. Recently, a large number of microsatellites, (CA)n repeats, have been identified from the equine genome [11, 14, 20, 23]. Many microsatellites are informative due to their high polymorphisms and they are therefore useful in paternity testing of horses such as native horses [4]. Although the use of them in parentage testing is useful for native horses, some microsatellites show few alleles and low heterozygosity for Thoroughbred horses. The parentage testing system for Thoroughbred horses showed a lower total PE value because of less polymorphic microsatellites.

In previous studies, we reported cloning and characterization of equine (CA)n and (CAG)n repeats from cosmid and enrichment libraries [25–30]. The cloned microsatellites were analyzed for polymorphisms using Thoroughbred horse individuals. Fifteen of the microsatellites showed PE values over 0.5.

Our objective was to construct a microsatellite set for parentage testing of Thoroughbred horses. In this study, we describe the set up of polymorphic microsatellites and a series of methods suitable for the routine paternity testing of Thoroughbred horses, and evaluated the constructed paternity testing system and methods using a large number of Thoroughbred horse individuals.

MATERIALS AND METHODS

Microsatellite loci studied: Microsatellite names, DDBJ, EMBL, and GenBank accession numbers, cloned repeat structures, primers, and PCR product sizes of the cloned microsatellites are given in Table 1.

Samples: Genomic DNAs were prepared from whole blood samples, which were collected from 250 unrelated Thoroughbred horses and 62 unrelated Asian native horses. Genomic DNAs from Thoroughbred horses were extracted using MagExtractor System MFX-2000 (Toyobo, Osaka, Japan) according to the manufacturer's protocols. Follow-

Table 1. Summary of fifteen equine microsatellite loci

Locus Accession No.	Structure Product size	Primer concentration	Fluorescence	Forward primer Reverse primer
TKY279	(CA)18	6 pmol	FAM-labele	AATGAATGAGACTTGAACCC
AB033930	127 bp	6 pmol	non-labele	TCTGCTGTTTTAGGCTCGG
TKY287	(CA)17...(CA)12	6 pmol	FAM-labele	ATCAGAGAACACCAAGAAGG
AB033938	230 bp	6 pmol	non-labele	TCTCTGCTATAGGTAAGGTC
TKY294	(CA)12GA(CA)5	5 pmol	NED-labele	GATCTATGTGCTAGCAAACAC
AB034603	221 bp	5 pmol	non-labele	CTAGTGTTTCAGATAGCCTC
TKY297	(CA)15	6 pmol	HEX-labele	GTCTTTTTGTGCCTCTGGTG
AB034606	228 bp	6 pmol	non-labele	TCAGGGGACAGTGGCAGCAG
TKY301	(CA)15	3 pmol	HEX-labele	AATGGTGGCTAATCAATGGG
AB034610	151 bp	3 pmol	non-labele	GTGTATGATGCCCTCATCTC
TKY312	(CA)3TA(CA)13	3 pmol	HEX-labele	AACCTGGGTTTCTGTTGTTG
AB034621	114 bp	3 pmol	non-labele	GATCCTTCTTTTTATGGCTG
TKY321	(CA)20	3 pmol	FAM-labele	TTGTTGGGTTTAGGTATGAAGG
AB034629	200 bp	3 pmol	non-labele	GTGTCAATGTGACTTCAAGAAC
TKY325	(AC)20TC(AC)4(GC)4	3 pmol	NED-labele	GGATGGAGTGAGATAATACC
AB044826	191bp	3 pmol	non-labele	TGGATGAACCATGAATAGTG
TKY333	(CA)19	6 pmol	NED-labele	CCTTCACTAGCCTTCAAATG
AB044834	107bp	6 pmol	non-labele	TTGTGTTTAGACAGTGCTGC
TKY337	(CA)15	3 pmol	HEX-labele	AGCAGGGTTTAATTACCGAG
AB044838	175bp	3 pmol	non-labele	TAGATGCTAATGCAGCACAG
TKY341	(AC)21	3 pmol	NED-labele	TATCCAGTCACCCATTTTAC
AB044842	156bp	3 pmol	non-labele	TTGTGTCAGTACACTCTATG
TKY343	(CA)5CT(CA)19(TA)5(CA)3	6 pmol	FAM-labele	TAGTCCCTATTTCTCCTGAG
AB044844	171bp	6 pmol	non-labele	AAACCCACAGATACTCTAGA
TKY344	(CA)20	6 pmol	FAM-labele	GTGTCCATCAATGGATGAAG
AB044845	109bp	6 pmol	non-labele	CTTAAGGCTAAATAATATCCC
TKY374	(CA)13	4 pmol	HEX-labele	CTGGTCCCTCTGGATGGAAG
AB044874	204bp	4 pmol	non-labele	TCCCAAGAGGGAGTACAATC
TKY394	(CA)17	5 pmol	NED-labele	GCATCATCGCCTTGAAGTTG
AB048299	244bp	5 pmol	non-labele	CCTTTCTGGTTGGTATCCTG

ing digestion with proteinase K (~2 μg/μl) in extraction buffer (10 mM Tris, 10 mM EDTA, 0.1 M NaCl, 2% SDS), genomic DNAs from Asian native horses were extracted by phenol/chloroform, ethanol precipitated, washed, and resuspended in sterile TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Fluorescent PCR: Labeled primers were prepared for multiplex PCR and analysis by one injection. Five microsatellites, *TKY279*, *TKY287*, *TKY321*, *TKY343*, and *TKY344*, were labeled with 6-FAM (Applied Biosystems, California, U.S.A.). Five microsatellites, *TKY297*, *TKY301*, *TKY312*, *TKY337*, and *TKY374*, were labeled with HEX (Applied Biosystems). Five microsatellites, *TKY294*, *TKY325*, *TKY333*, *TKY341*, and *TKY394*, were labeled with NED (Applied Biosystems) (Fig. 1). Multiplex PCR systems were performed for every fluorescent-label, thus 3 multiplex PCR systems were prepared. Each multiplex PCR was performed in a total volume of 20 μl of the following mixture: 20–50 ng of equine genomic DNA, each primer listed in Table 1, 200 μM of dNTPs, 2 μl of 10x reaction buffer; and 0.1 U of r*Taq* polymerase (Takara Shuzo, Kyoto, Japan). PCR amplification entailed initial denaturation (94°C, 4 min), 30 cycles of 1 min each at 94°C, 55°C and 72°C, and then 60 min at 72°C for final extension in a GeneAmp PCR System 9600 (Applied Biosystems). The final extension step at 72°C for 60 min promoted complete nontemplated 3'-nucleotide addition.

Genotype determination: Samples were prepared for electrophoresis and loaded onto ABI PRISM 310 Genetic Analyzer (Applied Biosystems). An internal lane size standard was included with every sample to allow automatic sizing of alleles. Samples run on ABI PRISM 310 Genetic Analyzer were prepared by combining each 0.5 μl of PCR product with 0.5 μl of GeneScan-350^{TM} [ROX] Internal Lane Size standard (Applied Biosystems), and 12 μl of deionized formamide. After denaturation for 5 min at 95°C and snap cooling, PCR products were injected electrokinetically (5 s, 15 kV) and electrophoresed at 15 kV in Performance Optimized Polymer 4 (POP4^{TM}) (Applied Biosystems). Data were collected using ABI PRISM 310 Collection Software application, version 1.0.2 (Applied Biosystems) with the GeneScan software run module 'GS POP4 (1 m*l*) D' (virtual filter set D). GeneScan software version 2.1 or 3.1 (Applied Biosystems) automatically analyzed the collected data and determined allele sizes by Local Southern method. Alleles were assigned by Genotyper 2.0 Software (Applied Biosystems). Alleles are designed with alphabetical symbols, in the order of smallest to largest, based on a middle-sized allele having been assigned as M.

Statistical analysis: Allele frequencies for each microsat-

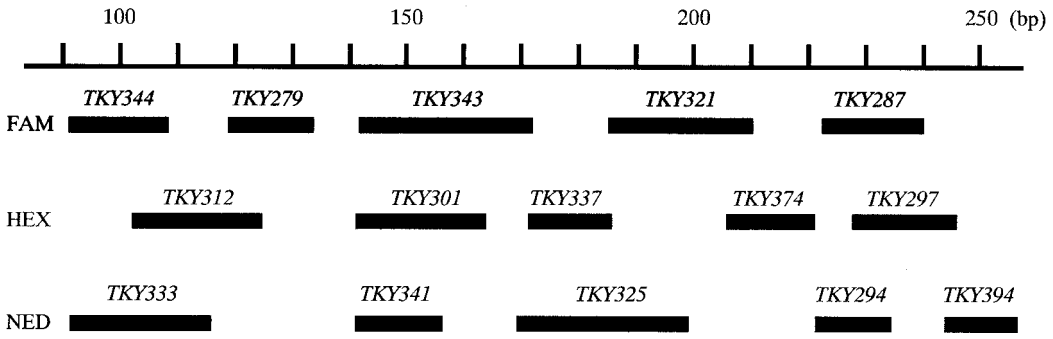

Fig. 1. Set up of the Thoroughbred parentage testing markers. We picked the 15 informative microsatellites for Thoroughbred parentage testing from previous studies [24–29]. The 15 microsatellites were selected based on PE values exceeding 0.5. Figure shows size range of the PCR products for Thoroughbreds. The 15 microsatellite loci can be analyzed in one lane using an ABI 310 Genetic Analyzer.

ellite were determined by direct counting from tests of 250 Thoroughbred horses and 62 Asian native horses. Observed heterozygosity (H_O) was determined by direct counting, and expected heterozygosity (H_E) was calculated from allele frequencies. Probability of exclusion (PE) was calculated from allele frequencies [17].

RESULTS

Fluorescent PCR assay: Fifteen microsatellites, *TKY279*, *TKY287*, *TKY294*, *TKY297*, *TKY301*, *TKY312*, *TKY321*, *TKY325*, *TKY333*, *TKY337*, *TKY341*, *TKY343*, *TKY344*, *TKY374*, and *TKY394* were selected for the construction of Thoroughbred horse-parentage testing system from microsatellites cloned in previous studies [25–30]. One of three different fluorescent dyes, each possessing a distinct emission spectrum, was employed so that five microsatellites of non-overlapping allele size range could be labeled with a single color (Fig. 1). Primers of four microsatellites, *TKY294*, *TKY321*, *TKY325*, and *TKY374*, were re-designed to set up the multiplex PCR systems. The newly designed primers are shown in Table 1. Each microsatellite was labeled with FAM, HEX, or NED. *TKY279*, *TKY287*, *TKY321*, *TKY343*, and *TKY344* were detected as blue; *TKY297*, *TKY301*, *TKY312*, *TKY337*, and *TKY374* were detected as green; *TKY294*, *TKY325*, *TKY333*, *TKY341*, and *TKY394* were detected as yellow. The incorporation of these three dyes allows the analysis of all 15 microsatellites by one injection after PCR amplification. This system was named Microsatellite 15 TKY System.

Three multiplex PCR amplifications were performed for each microsatellite set of the Microsatellite 15 TKY System. Figure 2 shows the results from amplification of genomic and control DNAs with the Microsatellite 15 TKY System. The microsatellites labeled with FAM (blue) are shown in Panel A, microsatellites labeled with HEX (green) are shown in Panel B and microsatellites labeled with NED (black) are shown in Panel C. All microsatellites of the Microsatellite 15 TKY System were well amplified. PCR products specific for each microsatellite were produced with some nonspecific amplification artifacts. The artifacts did not influence the PCR products specific for each microsatellite. These results indicated that the Microsatellite 15 TKY System would be useful for Thoroughbred horse parentage testing.

Genotyping of microsatellites: Allele sizes were automatically calculated by comparison with an in-lane size standard, labeled with a fourth dye, ROX (red). When each microsatellite cloned in vector was genotyped for the Microsatellite 15 TKY System, allele sizes of the microsatellites calculated by using the in-lane size standard were different from the allele-sizes obtained by sequencing of each microsatellite cloned in vector. Thus, true allele sizes were determined by comparing sample allele sizes to allele sizes of the cloned microsatellites calculated by the in-lane size standard.

Alleles were designated with alphabetical symbols from the above allele sizes. A middle-sized allele, assigned as M, was determined from allele data of Thoroughbred and Asian horse populations. The number of alleles/microsatellites of Thoroughbred and Asian horse populations varied from 5 to 9 and from 6 to 15, respectively. Asian horse population had alleles of wide range compared with Thoroughbred horse populations. All alleles of Thoroughbred and Asian horse populations were a series of alleles with differences of two base-pairs (bp); alleles with single bp differences were not found.

Allele frequencies: Random samples from two populations, 250 Thoroughbred and 62 Asian horses, were applied to type 15 microsatellites of the Microsatellite 15 TKY System. Allele frequency distributions of the two populations are shown in Table 2. A total of 624 chromosomes for all microsatellites of the populations were represented by 8 different alleles at *TKY279*, 12 at *TKY287*, 7 at *TKY294*, 11 at *TKY297*, 10 at *TKY301*, 12 at *TKY312*, 13 at *TKY321*, 14 at *TKY325*, 11 at *TKY333*, 7 at *TKY337*, 9 at *TKY341*, 15 at *TKY343*, 9 at *TKY344*, 10 at *TKY374*, and 8 at *TKY394*. Codominant inheritance of the 15 microsatellites was seen

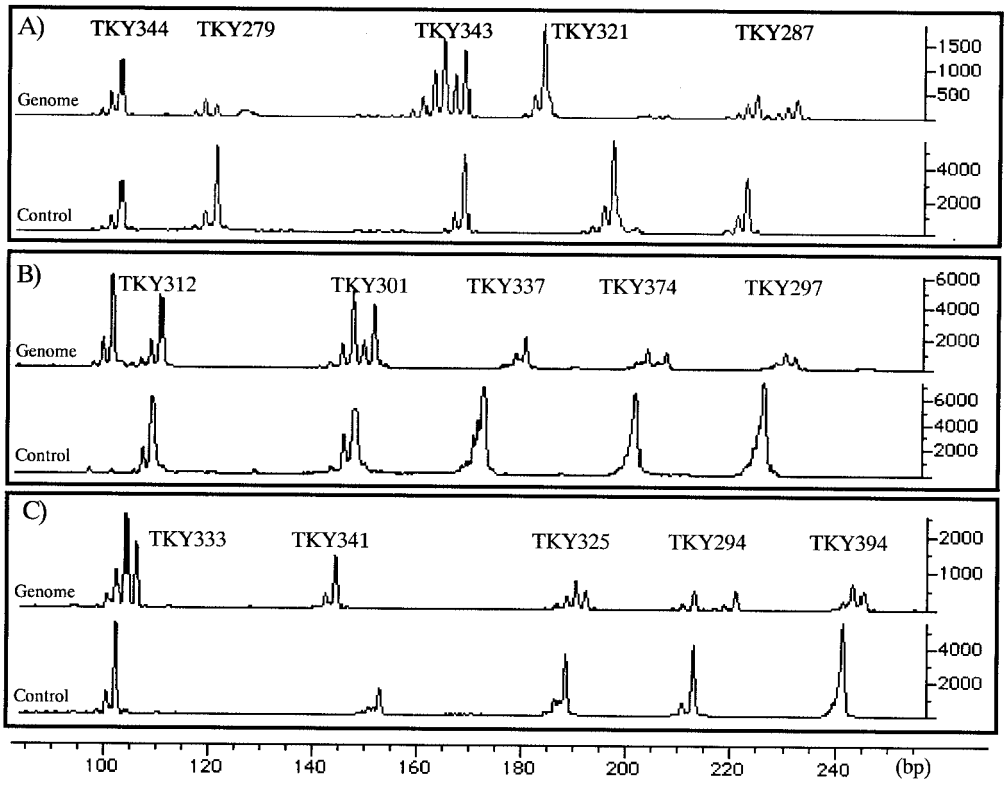

Fig. 2. Electrophoretic profile of an equine genome and cloned DNAs amplified using the Microsatellite 15 TKY System and detected using the ABI PRISM 310 Genetic Analyzer.

in one four-, two three-, and seventy-eight two-generation families as shown in Fig. 3. All microsatellites followed menderian inheritance with the Thoroughbred horse families.

Observed heterozygosity (H_O) and expected heterozygosity (H_E) of the 15 microsatellites for Thoroughbred horses were consistent, so there were neither null alleles nor an increase in homozygosity. In addition, these results explain the H-W equilibrium deviation (data not shown). It indicates that the allele frequency data are available for Thoroughbred horses. The high informativeness of the microsatellites used was demonstrated by the high number of detected alleles and 67–81% heterozygosity. The exclusion probability of each microsatellite was 55.2% (*TKY279*), 43.7% (*TKY287*), 49.9% (*TKY294*), 47.4% (*TKY297*), 52.3% (*TKY301*), 52.8% (*TKY312*), 62.1% (*TKY321*), 51.6% (*TKY325*), 57.3% (*TKY333*), 48.5% (*TKY337*), 48.5% (*TKY341*), 47.0% (*TKY343*), 56.7% (*TKY344*), 55.5% (*TKY374*), and 59.4% (*TKY394*) for Thoroughbred horses, and in a combination of the systems, 99.998% (Table 2).

Paternity testing for Thoroughbred horses: A case of paternity testing for a Thoroughbred horse family, which could not be determined by blood group system, was analyzed using the Microsatellite 15 TKY System (Fig. 4A). In this case, *TKY343* excluded paternity of sire 2 (S2) as shown in Fig. 4B. Foal (F) inherited allele "U" from dam (D), thus, the candidate sire must have allele "J", resulting in exclusion of sire 2 (S2).

DISCUSSION

Our objective in the present study was to construct a paternity testing system for Thoroughbred horses. Microsatellites suitable for this purpose are those showing multiple alleles as well as high heterozygosity. However, it should be emphasized that Thoroughbred horses, by definition, have much less genetic polymorphism and heterozygosity than humans and non-Thoroughbred horses, increasing the difficulty in the selection and set up of microsatellites useful for the purpose. In previous studies [25–30], we isolated a large number of microsatellites having high heterozygosity and a number of alleles. Because the microsatellites were isolated from Thoroughbred horse genomes and were genotyped using Thoroughbred horse individuals, polymorphism data of the microsatellites would be useful for construction of a parentage testing system for Thoroughbred horses. We selected microsatellites having a number of alleles and high heterozygosity based on the polymorphism data of previous studies [25–30].

Table 2. Provided for each microsatellites evaluated for Thoroughbred horse parentage testing are: allelic frequencies for Thoroughbred horses based on 250 horses and Asian horses based on 62 horses with M allele size (in base pairs) as defined by ABI 350ROX standards using the Local southern-size calling method in the Genescan Analysis software; observed (Ho) and expected (H_E) heterozygosity; and probability of exclusion (PE) and total PE calculated from allele frequency of Thoroughbred horse individuals

Loci	Populations	Alleles and frequencies																		H_O	H_E	PE
		B	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W			
TKY279	Thoroughbred						0.134			0.160	0.270	0.328	0.016	0.092							0.752	0.767	0.552
	Asian						0.194			0.105	0.194	0.161	0.145	0.161	0.024	0.016							
TKY287	Thoroughbred					0.002		0.196			0.156	0.152	0.002	0.002	0.486	0.004					0.648	0.678	0.437
	Asian					0.210	0.008	0.242	0.032	0.105	0.016	0.032	0.056	0.113	0.161					0.024			
TKY294	Thoroughbred						0.252			0.148	0.352	0.020	0.226	0.002							0.704	0.739	0.499
	Asian						0.032	0.056		0.121	0.371	0.129	0.290										
TKY297	Thoroughbred								0.242	0.124	0.340	0.290	0.004								0.640	0.726	0.474
	Asian						0.145	0.008	0.073	0.089	0.177	0.105	0.105	0.089	0.048	0.113	0.048						
TKY301	Thoroughbred							0.048	0.056	0.264	0.288	0.298	0.038			0.008					0.768	0.752	0.523
	Asian			0.008				0.081	0.040	0.097	0.218	0.121	0.242	0.008	0.008	0.177							
TKY312	Thoroughbred			0.028		0.136				0.396	0.240	0.018		0.140	0.042						0.720	0.745	0.528
	Asian			0.024		0.379	0.065	0.024	0.032	0.056	0.016	0.016	0.016	0.008	0.347	0.016							
TKY321	Thoroughbred					0.310			0.186	0.156		0.024		0.068	0.106	0.150					0.800	0.806	0.621
	Asian					0.040	0.065	0.016	0.048	0.105	0.242	0.032	0.210	0.024	0.016	0.129	0.065	0.008					
TKY325	Thoroughbred		0.038			0.096	0.122		0.022	0.034		0.338	0.346	0.002	0.002						0.760	0.739	0.516
	Asian	0.024	0.040	0.008	0.065	0.073	0.097	0.073	0.161	0.266	0.016	0.121	0.048		0.008								
TKY333	Thoroughbred						0.094	0.086						0.088	0.124	0.342	0.262	0.004			0.736	0.775	0.573
	Asian						0.056	0.234		0.177	0.016	0.065	0.048	0.040	0.097	0.081	0.177	0.008					
TKY337	Thoroughbred								0.322	0.276		0.258	0.138	0.006							0.728	0.735	0.485
	Asian						0.137		0.274	0.129	0.153	0.161	0.145										
TKY341	Thoroughbred					0.010	0.190	0.186	0.016	0.448	0.126	0.004		0.020							0.660	0.712	0.485
	Asian					0.024	0.161	0.137	0.169	0.387	0.024			0.089	0.008								
TKY343	Thoroughbred						0.020			0.390	0.118	0.016				0.124	0.004	0.326	0.002		0.700	0.712	0.470
	Asian		0.008	0.048	0.008	0.016	0.040	0.048		0.452	0.056	0.040		0.008	0.065	0.024	0.113	0.032	0.040				
TKY344	Thoroughbred					0.058		0.098		0.276		0.132	0.106	0.330							0.788	0.773	0.567
	Asian					0.008		0.350	0.056	0.105		0.081	0.040	0.274	0.065	0.016							
TKY374	Thoroughbred						0.080	0.100	0.120	0.288		0.348	0.030	0.034							0.752	0.763	0.555
	Asian						0.024	0.290	0.129	0.081	0.169	0.065	0.056	0.145	0.032	0.008							
TKY394	Thoroughbred						0.244	0.050	0.256	0.204		0.174	0.072								0.812	0.795	0.594
	Asian						0.169	0.089	0.210	0.153	0.081	0.121	0.040	0.137									
																				Total PE for Thoroughbred horses			0.99998

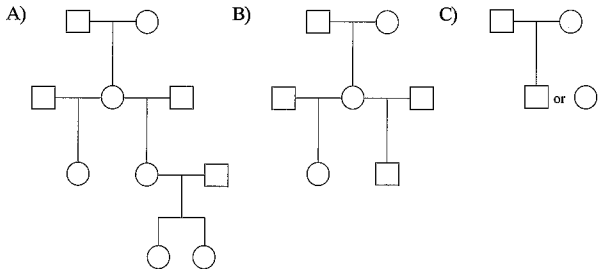

Fig. 3. One four- (A), two three- (B), and seventy-eight two-generation (C) Thoroughbred horse families were analyzed for segregation patterns of 15 microsatellites. All alleles of the microsatellites were correctly inherited to the next generation.

It has been a requirement of routine paternity testing to analyze by one injection after PCR amplification. Recently, multiple fluorescence has been utilized with a commercial automatic DNA sequencer. Even if the size range of each microsatellite overlaps, the use of different fluorescence enables simultaneous analyses of the overlapping microsatellites. The analysis of microsatellites with the automatic DNA sequencer generally shows a good resolution for PCR products ranging from 100 bp to 300 bp, resulting in a set up of multiplex PCR using about five microsatellites for every one fluorescence. Finally, we considered the set up of 15 microsatellites for one injection analysis by multiplex PCR, because the commercial automatic DNA sequencer can be used with three fluorescent dyes plus one for internal size markers. Because ISBC has demanded a higher PE value, more than 99.95% (0.9995), for paternity testing of Thoroughbred horses, a PE value of each microsatellite over 0.5 on average was required for a total PE value of over 0.9995 when using the 15 microsatellites. Thus, we selected 15 microsatellites having a PE value over 0.5, for Thoroughbred horse parentage testing, from previous studies [25–30]. Primers of four microsatellites were re-designed for the Microsatellite 15 TKY System. The Microsatellite 15 TKY System showed good resolutions for Thoroughbred horses, and delivered a total PE value of over 99.95%, indicating its usefulness for paternity testing of Thoroughbred horses. Recently, International Society of Animal Genetics (ISAG) presented 9 microsatellites for horse parentage testing. Because the total PE value of the 9 microsatellites was 99.91% (0.9991) for Thoroughbred horses, the Microsatellite 15 TKY System would supplement the total PE value.

There are many cases of paternity testing of Thoroughbred horses. Because one dam mates with multiple sires in one breeding season, it is necessary to exclude paternity of a false sire. Many microsatellites of the Microsatellite 15 TKY System excluded the paternity of a false sire in many cases. In addition, there were cases in which the candidate sires were a close relation as shown in Fig. 4A. In this par-

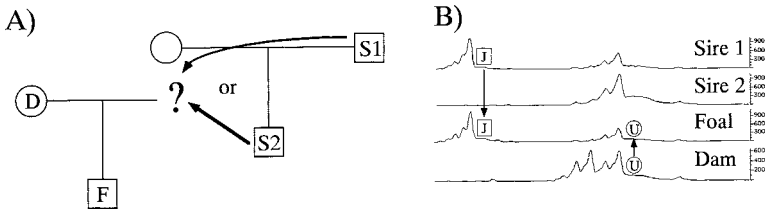

Fig. 4. Paternity testing case of Thoroughbred horse family. A) Candidate sires were a close relation. B) Paternity testing results of Thoroughbred horse family shown in Fig. 4A by *TKY343* locus in the Microsatellite 15 TKY System. *TKY343* excluded paternity of sire 2; foal inherited allele "U" from dam, thus, the candidate sire must have allele "J".

ticular case, it was necessary for exclusion of the paternity that each allele of the candidate sires excepting inherited alleles were different alleles from each other, and that the dam should not have the different alleles. Microsatellites for this case required a large number of alleles, high heterozygosity, and equal frequency of each allele, and the Microsatellite 15 TKY System succeeded in exclusion of a paternity. These results indicate the Microsatellite 15 TKY System is a powerful tool for Thoroughbred horse parentage testing.

Replication slippage is detected as stutter bands of lower intensity and is smaller than the actual allele size (Fig. 4). This stutter band problem is the major disadvantage of using microsatellites in parentage testing due to the risk of wrong allele typing. In particular, mono- and dinucleotide repeats, such as (CA)n repeats, show stutter bands. In this study, almost all microsatellites showed stutter bands, resulting in difficulty of automatic analysis by computer software. Strand misparing by slippage is thought to be the major mechanism for producing stutter bands at dinucleotide repeats [10]. Due to this, the general rule to identify true alleles is to identify the band showing the strongest signal and the band of the slowest electrophoretic mobility. By using these criteria, we analyzed the segregation of one four-, two three-, and seventy-eight two-generation families. All alleles of the microsatellites were correctly inherited to the next generation. Thus, we concluded that the stutter bands did not interfere with the correct reading of identification in a series of trials using the Microsatellite 15 TKY System.

Microsatellites used for equine parentage testing in this study showed greater variability than blood typing systems. This is due to non-coding DNA; they are not a part of genes that have a function. Nozawa *et al.* (1998) [16] revealed a phylogenetic relationship of east-Asian native horses using polymorphisms of blood group proteins. However, some markers did not show the polymorphisms, resulting in less information. The microsatellites of the Microsatellite 15 TKY System showed a number of alleles and high heterozygosity for Thoroughbred horses as well as Asian native horses. Thus, these microsatellites might be useful for population genetics of the horse.

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