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Validation of 17 Microsatellite Markers for Parentage Verification and Identity Test in Chinese Holstein Cattle

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ABSTRACT : To develop an efficient DNA typing system for Chinese Holstein cattle, 17 microsatellites, which were amplified in four fluorescent multiplex reactions and genotyped by two capillary electrophoresis injections, were evaluated for parentage verification and identity test. These markers were highly polymorphic with a mean of 8.35 alleles per locus and an average expected heterozygosity of 0.711 in 371 individuals. Parentage exclusion probability with only one sampled parent was approximately 0.999. Parentage exclusion probability when another parent's genotype was known was over 0.99999. Overall probability of identity, i.e. the probability that two animals share a common genotype by chance, was 1.52×10^{-16}. In a test case of parentage assignment, the 17 loci assigned 31 out of 33 cows to the pedigree sires with 95% confidence, while 2 cows were excluded from the paternity relationship with candidate sires. The results demonstrated the high efficacy of the 17 markers in parentage analysis and individual identification for Chinese Holstein cattle. (**Key Words :** Parentage Analysis, Identity Test, Microsatellite, Multiplex PCR, Chinese Holstein)

INTRODUCTION

The Holstein is the most important dairy cattle breed in the world as well as in China. In past decades, its performance has been genetically improved significantly. Genetic evaluation, which plays a key role in a genetic improvement program, requires accurate pedigree information. In practice, however, the proportion of pedigree error has been estimated at 3 to as high as 23% in the Holstein population in some countries (Ron et al., 1996; Visscher et al., 2002; Weller et al., 2004; Sanders et al., 2006). Incorrect paternity will consequently lead to biased estimates of heritability and reduced genetic gain (Visscher et al., 2002; Weller et al., 2004; Sanders et al., 2006).

Traditionally, pedigree verification in dairy cattle has been carried out using blood groups. During the past decade, DNA typing based on microsatellite markers has become the international standard system of parentage verification and identity testing in livestock (ISAG Conference, 2006). For genotyping in bovines, the Food and Agriculture Organization of the United Nations (FAO) initially recommended 30 microsatellite loci for genetic diversity studies (FAO/ISAG, 1993), which were updated in 2004 (FAO/ISAG, 2004). Later, the International Society of Animal Genetics (ISAG) suggested a panel of 9 loci (*BM1824*, *INRA23*, *BM2113*, *SPS115*, *ETH10*, *TGLA122*, *ETH225*, *TGLA126* and *TGLA227*) to be used in cattle parentage analysis (ISAG Conference, 2006). Recently, three new loci (*BM1818*, *ETH3* and *TGLA53*) were added to the ISAG recommended panel (ISAG Conference, 2008). These loci were demonstrated with high polymorphism by many studies (e.g. Curi and Lopes, 2002; Herráez et al., 2005; Radko et al., 2005; Rahimi et al., 2006; Rehout et al., 2006; Ozkan et al., 2009). Meanwhile, the recommended loci were shown effective in parentage analysis and identity testing, with estimations of >0.99 of total probability of exclusion (Curi and Lopes, 2002; Rahimi et al., 2006; Ozkan et al., 2009) and $<10^{-8}$ of probability of identity (Herráez et al., 2005).

In China, for a long time the breeding stocks of Holstein were largely selected from North America and Europe in the form of live bulls or embryos. To identify genetically superior bulls, the Ministry of Agriculture and Dairy Association of China (DAC) recently launched a nation-wide genetic improvement program aiming to establish a progeny-testing system for dairy cattle in China. DNA-based parentage analysis is required for pedigree

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verification in the progeny test. Our previous study (Tian et al., 2008) investigated the power of microsatellite markers for paternity testing in Chinese Holstein cattle. However, the genotyping method used in that study, polyacrylamide gel electrophoresis combined with silver-stained testing, was extremely time consuming and the precision of genotyping was low; these deficiencies prevented its convenient application in routine testing. The objective of the current study was to develop a fluorescent genotyping system using highly polymorphic microsatellite markers and to assess its usefulness in parentage verification and individual identity test in Chinese Holstein cattle.

MATERIALS AND METHODS

Animals

A total of 371 Chinese Holstein cattle samples were genotyped. Blood samples of 157 cows were collected from 10 different dairy herds in Beijing. Semen samples of 214 bulls were obtained from 7 bull centers in China, which were scattered across locations in 7 provinces or regions of China. Genomic DNA of blood samples was extracted by a standard proteinase K digestion followed by phenol/chloroform extraction (Sambrook et al., 1989). For semen samples, β-mercaptoethanol was added to the lysis buffer.

Microsatellite loci

Seventeen bovine microsatellites that have been mapped to sixteen different autosomes were employed in this study (Table 1). Selection criteria included recommendation of ISAG (ISAG Conference, 2008) and FAO (FAO/ISAG, 2004), polymorphism of markers tested by our previous study (Tian et al., 2008), and size range of markers suitable for grouping into a fluorescent genotyping system.

Multiplex PCR and genotype determination

Based on prior information of PCR product size, the 17 loci were grouped into two sets, consisting of 10 and 7 loci, respectively. Loci in each set could be simultaneously separated by one capillary electrophoresis injection, according to the size and the fluorescent color of PCR products. Moreover, within each set, markers were classed into multiplex PCR groups to increase throughput using the MultiPLX version 2.0 software (Kaplinski et al., 2005). Multiplex groups were automatically obtained by estimating the compatibility of primers, such as primer-primer interactions, primer-product interactions, difference in melting temperatures, and the risk of generating alternative products from the template (Kaplinski et al., 2005).

The forward primer of each locus was end-labeled with fluorescent dye (6-FAM, VIC or HEX) (Table 1). The optimization of amplification was implemented by the procedure of Zhang et al. (2008). Amplifications were performed in a thermocycler 9700 GeneAmp® (Applied Biosystems). Capillary electrophoresis was performed in an

Table 1. Microsatellite marker sets, locus, size range, fluorescent dye, multiplex PCR and capillary electrophoresis injection

	Locus	Size	Dye	Multiplex PCR[3]	Electrophoresis injection [4]
10 marker set	*ETH10* [2]	208-224	HEX	A	I
	ETH225 [2]	137-156	HEX	A	I
	TGLA227 [2]	79-104	6-FAM	A	I
	BM1818 [2]	256-268	6-FAM	A	I
	TGLA126 [2]	116-126	HEX	A	I
	BM1824 [2]	176-190	HEX	B	I
	INRA23 [2]	197-215	6-FAM	B	I
	TGLA53 [2]	150-172	6-FAM	B	I
	BM2113 [2]	123-137	6-FAM	B	I
	TGLA122 [2]	138-183	VIC	B	I
17 marker set[1]	*MM12*	110-128	HEX	C	II
	HEL9	146-169	6-FAM	C	II
	INRA063	174-184	HEX	C	II
	SPS115 [2]	245-257	6-FAM	D	II
	ILSTS006	284-296	HEX	D	II
	ETH152	189-205	6-FAM	D	II
	CSRM060	90-102	6-FAM	D	II

[1] In addition to the 10 marker set. [2] ISAG recommended microsatellite for cattle paternity testing.

[3] Markers with the same letter were grouped into one multiplex PCR set.

[4] PCR products of multiplex A and multiplex B were mixed for capillary electrophoresis injection I; PCR products of multiplex C and multiplex D were mixed for capillary electrophoresis injection II.

Table 2. Number of alleles (NA), observed heterozygosity (H_O) and expected heterozygosity (H_E), polymorphic information content (PIC) for seventeen markers in Chinese Holstein cattle

Locus	NA	H_O	H_E	PIC
BM1818	7	0.601	0.661	0.599
BM1824	6	0.742	0.757	0.713
BM2113	7	0.735	0.761	0.726
ETH10	8	0.609	0.681	0.635
ETH225	8	0.778	0.771	0.734
INRA23	9	0.761	0.767	0.730
TGLA122	16	0.757	0.803	0.778
TGLA126	6	0.632	0.656	0.591
TGLA227	12	0.771	0.826	0.808
TGLA53	11	0.712	0.817	0.791
CSRM060	6	0.639	0.661	0.624
ETH152	7	0.734	0.727	0.691
HEL9	12	0.827	0.828	0.804
ILSTS006	7	0.689	0.678	0.618
INRA063	5	0.525	0.554	0.454
MM12	8	0.560	0.567	0.493
SPS115	7	0.546	0.580	0.542
Mean	8.35	0.683	0.711	0.667

ABI PRISM Genetic Analyzer 3730 (Applied Biosystems) according to the manufacturer's recommendations. Genotyping data were analyzed with GeneMapper® version 3.0 software (Applied Biosystems) and sized according to the internal lane size standard (GeneScan™-500 LIZ®, Applied Biosystems).

Statistical methods

The measures of genetic variability, including the number of alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E), and polymorphic information content (PIC) (Botstein et al., 1980), were calculated for each locus. Probability of exclusion (PE) was defined for three cases (Jamieson and Taylor, 1997). PE_1 estimates the probability of exclusion of a parent when genotypes of the offspring and two parents are known; PE_2 estimates the probability of exclusion of parentage relationship when genotypes of the offspring and one parent is known; and PE_3 estimates the probability of excluding the putative pair of parents when genotypes of the offspring and two parents are known. Cumulative probabilities of exclusion over *n* unlinked loci in all three of the above cases were also calculated according to Jamieson and Taylor (1997). All the calculations were performed with the CERVUS 3.0 software (Kalinowski et al., 2007). The probability of identity (PI) is the probability that two randomly chosen individuals in a population have identical genotypes; this was computed based on allele frequencies and using the GenAlEx version 6 program (Peakall and Smouse, 2006). Multilocus overall PI values were obtained by multiplying single-locus PI values, assuming independence of microsatellites. In addition, parentage inference was performed on a sample of 3 half-sib sire families. Likelihood based parentage analysis was performed using the CERVUS 3.0 software (Kalinowski et al., 2007).

RESULTS AND DISCUSSION

Multiplex PCR

According to the automatic grouping of PCR primers by MultiPLX software (Kaplinski et al., 2005), two five-plex sets were obtained from the first ten loci, while one quadplex and one triplex were developed from the later seven loci (Table 1). Thus, the genotype of markers could be determined by four multiplex reactions followed by two capillary electrophoresis injections (Figure 1). The testing efficiency of the current study was significantly improved in comparison with the genotyping method reported by Tian et al. (2008). Firstly, the testing time consumed by the new system was about 1/20~1/10 of that for the silver-stained testing system, mainly due to adapting PCR multiplexing and multicolor capillary electrophoresis techniques. Secondly, the precision of allele sizing was increased. The individual genotype in fluorescent-based detection systems is determined by reference to an internal lane standard, while in the silver-stained system by reference to size marker in adjacent lanes of the gel. Therefore, the 17 microsatellite typing system developed in this study is eligible for routine DNA testing of cattle in China.

Polymorphism, probability of exclusion and probability of identity

The number of alleles per locus (NA) varied from 6 (*TGLA126*) to 16 (*TGLA122*). The mean NA across 17 loci was 8.35. The expected heterozygosity (He) ranged from 0.554 (*INRA063*) to 0.828 (*HEL9*). Among the tested 17 loci, *TGLA227*, *HEL9*, *TGLA53*, *TGLA122*, *ETH225*, *INRA023*, *BM2113*, *BM1824*, showed higher polymorphism with PIC values higher than 0.7. These estimations were generally similar to those reported by Herráez et al. (2005), Rahimi et al. (2006), Řehout et al. (2006) and Ozkan et al. (2009).

The high genetic variability of markers implied their high effectiveness for parentage testing. The cumulative PE is a measure of the ability of a certain panel of marker to identify genetic paternity, excluding all other candidates. The probability of exclusions, PE1, PE2 and PE3 as defined in the Materials and Methods section, are shown in Table 3. As for PE1 and PE3 measures, the cumulative values were >0.999, regardless of whether all 17 loci or only 10 loci were considered. However, for the case that the genotype of a confirmed parent is unknown (i.e., the case of PE_2), the

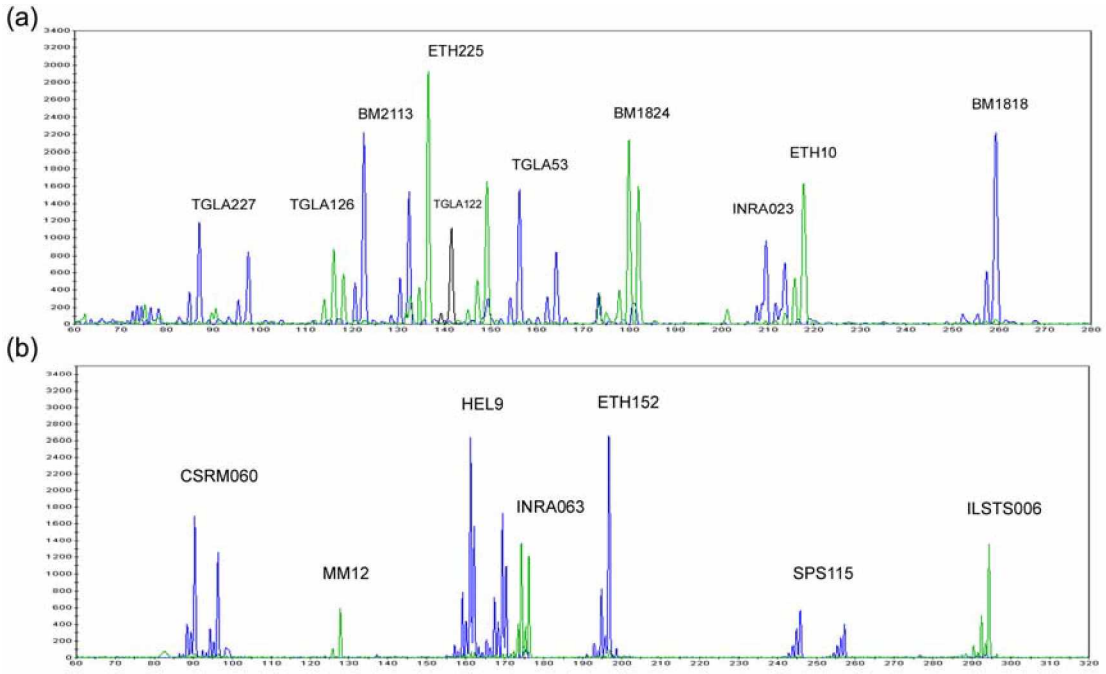

Figure 1. Electrophoresis profiles of a Chinese Holstein cattle sample (a) on the first 10 markers and (b) on the second seven markers.

expanded marker set (17 marker set) showed substantial higher cumulative PE value (0.999) than the 10 marker set (0.990). These values were higher than the parentage testing power of the system developed by Tian et al. (2008), and the systems for Iranian Holstein bulls (Rahimi et al., 2006), Czech (Rehout et al., 2006) and Turkish (Ozkan et al., 2009) Holstein populations using approximately 10 markers.

The 10 marker set in our system could produce similar exclusion probabilities to the commercially available StockMarks® kit (Applied Biosystems) in which 11 microsatellite markers are included. However, the additional 7 markers in the current study substantially expanded the power and would be useful especially for situations in which there are many candidate parents and no known parent is available.

The probability of two random animals having identical genotypes was estimated at 6.34×10^{-11} and 1.52×10^{-16} for the 10 and 17 loci set, respectively. Even in the extreme situation that all individuals were in full-sib relationships, the probability of identity was 1.04×10^{-4} and 4.68×10^{-7}, respectively, for the two marker sets (Table 3). That is, even with close relatedness among animals, this microsatellite panel is theoretically sufficient for individual identification of any cattle in the Chinese Holstein breed.

Practical validation for parentage assignment

Although the high PE estimation theoretically represents high effectiveness in paternity analysis, the effective exclusion probability in a given case will vary with the genotype of candidates and relatedness among the candidates. In this study, a sample of 33 cows and 3 candidate sires was chosen to evaluate the power of the test. The parameters used for likelihood based parentage analysis in CERVUS 3.0 software were as follows: 10,000 offspring, 3 candidate parents, 90% loci typed, 1% of genotyping error rate, 80% of relaxed confidence and 95% of strict confidence. Paternity analysis was carried out using the 17 and 10 marker sets. Two individuals were found with incompatibility at more than two loci with the 3 candidate sires, probably due to pedigree error. With 17 loci, the non-excluded 31 offspring were all assigned parentage to the pedigree sires with 95% confidence. The delta value (Δ),

Table 3. The cumulative probability of exclusion and Multi-locus probability of identity estimations for two marker sets

	Cumulative probability of exclusion[1]			Overall probability of identity[2]	
	PE_1	PE_2	PE_3	PI	PI-Sib
10 marker set	0.999615	0.990039	0.999998	6.34×10^{-11}	1.04×10^{-4}
17 marker set	0.999993	0.998922	>0.999999	1.52×10^{-16}	4.68×10^{-7}

[1] PE_1 = Probability of exclusion (both parents known); PE_2 = Probability of exclusion (only one parent is known); PE_3 = Probability of exclusion (both parents known, exclude two putative parents).

[2] PI = Average probability that two randomly chosen individuals have identical genotypes; PI-Sib = Average probabilities that two full siblings have identical genotypes.

which is a statistic to evaluate the confidence of parentage assignments (Kalinowski et al., 2007) and computed for each pair of a progeny and its candidate sire in the CERVUS 3.0 software, ranged from 2.67 to 10.80. With 10 loci, however, the delta value (Δ) varied from 1.12 to 8.33, and only 24 progeny were assigned with 95% confidence. This highlighted the importance and necessity of the additional seven loci for the new system to achieve a high efficiency of testing.

In conclusion, the current study developed a convenient and efficient fluorescent typing system involving seventeen microsatellites for routine individual identification and parentage testing in the Chinese Holstein population.

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