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Validation of Microsatellite Markers for Parentage Verification in Indian HF Cattle

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A B S T R A C T

The study was undertaken with an objective to develop and validate a panel containing maximum number of microsatellite markers that can be amplified in a single PCR reaction for precise parentage verification in Indian HF cattle population. The study was based on a total of 210 HF cattle (100 calf, 100 dam and 10 sires). Genomic DNA was extracted from blood and semen samples. A panel of 12 microsatellite markers (BM1824, BM2113, INRA023, SPS115, TGLA122, TGLA126, TGLA227, ETH10, ETH225, BM1818, ETH3, TGLA53) was amplified in a single multiplex reaction and analyzed by capillary electrophoresis on an automated DNA sequencer. The observed heterozygosity (Ho) of 12 markers ranged from 0.607 (TGLA53) to 0.904 (BM2113) while the expected heterozygosity (He) ranged from 0.581 (ETH225) to 0.873 (INRA23). Eleven out of 12 microsatellite loci revealed relatively high polymorphic information content (>0.6). The results suggest that multiplex microsatellite panel can be effectively used to verify the parentage as well as to assign the putative sire to daughters under progeny testing.

Introduction

To achieve efficient implementation of the breeding programs that include selective reproduction it is necessary to have errorless pedigree information and correct relationships between the animals in the population. The Holstein is the most important dairy cattle breed in the world as well as in India. 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. The identification of proven sires has been of utmost importance in animal improvement programmes. Failure to record correct parentage can cause bias in sire evaluation, by introducing errors in estimates of heritabilities and breeding values. Misidentification reduces genetic gain with sire models (Gelderman *et al.*, 1986) and may have an even greater effect with animal models that account for all assumed genetic relationships (Wiggans *et al.*, 1988).

In the past, parentage testing in cattle has been carried out through the blood group and the protein polymorphism analysis, but because of some drawbacks, these tests have been replaced with new ones that are based on detection of certain "genetic markers". There are different approaches based on DNA polymorphism for paternity testing including RFLPs (Kashi *et al.,* 1990), multilocus, minisatellite and oligosynthetic probes (Trommelen *et al.,* 1993), and PCR based amplification of minisatellites and microsatellites (Schnabel *et al.,* 2000). However, RFLPs generally suffer from low heterozygosities and low PIC, while the DNA fingerprints are difficult to interpret owing to complex nature of banding pattern revealed.

The development of DNA technology has opened a new possibility for developing more sophisticated and more accurate methods that are based on DNA analysis. Most informative and most commonly used are the microsatellite markers (Short Tandem Repeats) which are highly polymorphic and are located on the noncoding intron regions of the bovine genome. Any sample containing nuclear DNA can be used for analysis.

The objective of the present study was to check the efficacy of microsatellite markers and its usefulness in parentage verification and individual identity test in Indian Holstein cattle.

Materials and Methods

Sample collection

Experimental material for the present study comprised of 210 samples of cattle (100 dams, 100 daughters, and 10 sires) of field progeny testing program conducted by National Dairy Development Board. 5 ml of blood sample and 2 frozen semen doses of sires used in program were transferred to laboratory under cold chain.

DNA extraction

Extraction of good quality high molecular weight genomic DNA is the prime requirement for the success of molecular genetic research. Therefore, the first step in genomic studies is the isolation of high molecular weight DNA. Genomic DNA was extracted from the blood samples by using QIAamp Kit (QIAGEN, USA) following manufacturer's instructions. The DNA from semen samples was extracted using QIAamp® DNA Investigator Kit (QIAGEN). The genomic DNA concentration ranged from 40-70 ng/µl. The quality and purity of DNA were checked by UV spectrophotometry. Most of the DNA samples had the O.D. ratio in the range of 1.8 to 2.0 (OD 260: 280).

Multiplex PCR optimization

To establish high throughput microsatellite analysis, we placed ISAG (International Society for Animal Genetics) recommended 12 microsatellite markers (Table 1), arranged by fragment size and fluorescent dye label, into a single PCR multiplex panel. Multiplex PCR of genomic DNA requires optimization of annealing temperature which decides the net synthesis of product during thermal cycling. Hence, to get the amplification of all used microsatellite oligos, optimization of reaction conditions were done by using different combinations of oligos and different annealing temperatures. Different annealing temperatures were tested from a range of 55-65^0 C in the mastercycler gradient and consistent results were obtained at 57 ^{0}C for all the primers when amplified in single multiplex panel.

Multiplex PCR amplification

The multiplex panel of 12 primers used in the present study successfully amplified 78-288bp PCR products.

Fragment analysis

In the present investigation, Parentage verification of HF pedigrees was attempted using single Multiplex PCR panel consisting of 12 microsatellite markers in a simple and highly accurate method. The present study provides a more reliable and useful method for extensive verification of cattle pedigrees. The amplified multiplex PCR products were diluted 8 times to obtain optimum peak height. Each 1µl of PCR product was mixed with 0.3 µl of size standard fluorescent dye GS Liz 500 (PE- Applied Biosystems) and finally made the volume up to 10 µl with Hi-Di formamide. Samples were denatured for 5 min at 95 ^{0}C and snap chilled on ice for 5 minutes before being run on ABI-3500 XL genetic analyzer

Gene scan analysis of microsatellites

The fluorescently end-labeled microsatellite PCR products were run on ABI 3500 XL capillary based genetic analyzer and analyzed in the presence of GS Liz 500 molecular weight standards labeled with the fluorescent dye Liz (PE- Applied Biosystems). The raw data were collected using ABI 3500 Data collection Software Version 1.0 Microsatellite fragment sizing was performed by the Gene Mapper version 4.1 (Applied Biosystems). Size standard peaks were defined by the user. Allele calling was performed with the software and were checked manually to avoid any false calling of alleles.

Statistical analysis

The data for all the samples were analyzed for genetic variability parameters such as allele counts, frequencies, and observed and expected heterozygosity. Dam-daughter-sire trio was verified with respect to Mendelian inheritance. Every daughter should show Mendelian inheritance of microsatellite alleles (i.e., every allele of a daughter should be traced back to either dam or sire). Parentage data was verified manually by matching alleles at 12 microsatellite loci in the daughter with that of dam and sire. Exclusion probability was estimated using formula given by Chakravarti and Li (1983) and Jamieson (1994) and modified by Marshall *et al.,* (1998). Combined exclusion probability takes account of individual exclusion probability and was calculated by following formula:

$$P = 1 - \prod_{i=1}^{n}(1 - Pi)$$

In which n = number of markers, Pi = individual exclusion probability for single marker,
II = Symbol of product, and P = combined exclusion probability.

Results and Discussion

Allele frequency

The number of alleles per locus (k) varied from 7 (ETH225 and ETH3) to 16 (INRA23). The mean number of alleles across 12 loci was 10.5. The allele range (7 to 16) corresponded to those determined previously, which ranged from 4 to 16 (Peelman *et al.,* 1998; Maudet *et al.,* 2002; Visscher *et al.,* 2002; Machado *et al.,* 2003; Sodhi *et al.,* 2006; Van Eennenaam *et al.,* 2007; Stevanovic *et al.,* 2010; Zhao *et al.,* 2017). It should be noted that the animals used in the study are related and higher number of alleles will be expected if larger samples of unrelated cattle are used (Table 2).

Observed (Ho) and Expected Heterozygosity (He)

All 12 markers were highly informative and complied with the prerequisites for parentage verification which requires expected heterozygosity (Hexp) and polymorphic information content (PIC) values of above 0.5 (Marshall *et al.,* 1998). The observed

heterozygosity (Ho) of 12 markers ranged from 0.607 (TGLA53) to 0.904 (BM2113). The expected heterozygosity (He) ranged from 0.581 (ETH225) to 0.873 (INRA23). The He and Ho range corresponded to those reported previously (Peelman *et al.*, 1998; Maudet *et al.*, 2002; Visscher *et al.*, 2002; Machado *et al.*, 2003; Sodhi *et al.*, 2006; Van Eennenaam *et al.*, 2007; Stevanovic *et al.*, 2010; Zhao *et al.*, 2017).

Table.1 Microsatellite markers used to verify Parentage in HF cattle

Locus		Primer Sequence (5'-3')	Dye	Primer range in bp
BM1824	Forward	GAG CAA GGT GTT TTT CCA ATC	NED	170-218
	Reverse	CAT TCT CCA ACT GCT TCC TTG		
BM2113	Forward	GCT GCC TTC TAC CAA ATA CCC	6 FAM	116-146
	Reverse	CTT CCT GAG AGA AGC AAC ACC		
INRA023	Forward	GAG TAG AGC TAC AAG ATA AAC TTC	6 FAM	194-236
	Reverse	TAA CTA CAG GGT GTT AGA TGA ACT C		
SPS115	Forward	AAA GTG ACA CAA CAG CTT CTC CAG	6 FAM	240-288
	Reverse	AAC GAG TGT CCT AGT TTG GCT GTG		
TGLA122	Forward	CCC TCC TCC AGG TAA ATC AGC	VIC	133-193
	Reverse	AAT CAC ATG GCA AAT AAG TAC ATA		
TGLA126	Forward	CTA ATT TAG AAT GAG AGA GGC TTC T	VIC	104-132
	Reverse	TTG GTC TCT ATT CTC TGA ATA TTC C		
TGLA227	Forward	CGA ATT CCA AAT CTG TTA ATT TGC T	6 FAM	63-115
	Reverse	ACA GAC AGA AAC TCA ATG AAA GCA		
ETH10	Forward	GTT CAG GAC TGG CCC TGC TAA CA	6 FAM	198-234
	Reverse	CCT CCA GCC CAC TTT CTC TTC TC		
ETH225	Forward	GAT CAC CTT GCC ACT ATT TCC T	6 FAM	132-166
	Reverse	ACA TGA CAG CCA GCT GCT ACT		
BM1818	Forward	AGCTGGGAATATAACCAAAGG	VIC	248-272
	Reverse	AGTGCTTTCAAGGTCCATGC		
ETH3	Forward	GAACCTGCCTCTCCTGCATTGG	NED	89-131
	Reverse	ACTCTGCCTGTGGCCAAGTAGG		
TGLA53	Forward	GCTTTCAGAAATAGTTTGCATTCA	6 FAM	147-197
	Reverse	ATCTTCACATGATATTACAGCAGA		

Table.2 Variability measures of 12 microsatellite markers in Indian HF cattle

Sr. No.	Locus	(k)	(Ho)	(He)	(PIC)	(EP)
1	BM1818	13	0.823	0.861	0.845	0.434
2	BM1824	9	0.828	0.79	0.757	0.589
3	BM2113	14	0.904	0.838	0.82	0.476
4	ETH10	14	0.833	0.853	0.836	0.446
5	ETH225	7	0.656	0.581	0.549	0.806
6	ETH3	7	0.617	0.67	0.612	0.748
7	INRA23	16	0.799	0.873	0.859	0.404
8	SPS115	10	0.722	0.806	0.778	0.555
9	TGLA122	8	0.703	0.717	0.668	0.694
10	TGLA126	8	0.679	0.698	0.666	0.696
11	TGLA227	10	0.727	0.78	0.744	0.606
12	TGLA53	10	0.607	0.691	0.661	0.699

Where, (k): Number of alleles at the locus, (Ho): Observed heterozygosity, (He): Expected heterozygosity, PIC: Polymorphic information content, (EP): Exclusion Probability

Polymorphic Information Content (PIC)

Among the tested 12 loci, TGLA227, BM2113, TGLA53, ETH10, TGLA126, TGLA122, INRA23, BM1818, ETH3, ETH225, BM1824 showed higher polymorphism with PIC values greater than 0.6. 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.

Exclusion Probability (EP)

The EP value was greatest for marker SPS115 (0.806) and least for marker TGLA122 (0.404). Combined EP for the selected 12 markers was 0.9985 indicating parentage assignments with 99% of confidence.

The cumulative exclusion probability is a measure of the ability of a certain panel of marker to identify genetic paternity, excluding all other candidates. These estimations were generally similar to those reported by Cervini *et al.,* (2006), Rehout *et al.,* (2006), Riojas-Valdes *et al.,* (2009), Van Eenennaam *et al.,* (2007).

Parentage verification

Parentage was verified for each daughter likewise by comparing daughter dam–sire trio and marked as correct or wrong. We found 4 daughters having wrong parentage out of 100 daughters using the above procedure. The results of this study showed a relatively low pedigree error rate of 4 %. Christensen et al (1982) reported misidentification rates between 5 and 15% in Danish dairy cattle, Geldermann *et al.,* (1986) estimated misidentification rates of 13% using blood group factors and biochemical polymorphisms in cattle. Ron et al (1996) found a 5% misidentification rate using microsatellite analysis in Israeli dairy cattle. Rosa (1997) reported a misidentification rate of 15% in Brazilian livestock, based on restriction fragment length polymorphism (RFLP) and microsatellite analysis.

From present study it can be concluded that a multiplex microsatellite panel consisting of 12 loci has been successfully validated for its use in paternity studies in Indian HF cattle. It is a fast, robust, reliable, and economic tool to verify the parentage as well as to assign the

putative sire to daughters under progeny testing program with very high accuracy.

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