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APPLICATION OF EIGHTEEN MICROSATELLITE MARKERS IN STUDIES PARENTAGE TESTING AND GENETIC DIVERSITY IN HOLSTEIN CATTLES

ROYA ASHOORY [1], CYRUS AMIRINIA [*2], ALIREZA NOSHARY [3], HAMIDREZA SEYDABADI [4]

[1, 3] Faculty of Agriculture and Natural Resources , Karaj Branch , Islamic Azad University, Karaj, IRAN

[2, 4] Department of Biotechnology, Animal Science Research Institute of Iran , Karaj ,IRAN

Corresponding author: amirinia@asri.ir *

ABSTRACT

Over the last few decades, the use of molecular markers has played an increasing role in studies parentage testing and genetic diversity within and among population. Microsatellites have been utilized most extensively, because they can be readily amplified by PCR and the large amount of allelic variation at each locus. These markers are abundant, distributed throughout the genome and are highly polymorphic compared with other genetic markers, as well as being species-specific and co-dominant. In this study, we used 18 microsatellite markers to investigate them polymorphism and them power of detection in parentage testing. A total of DNA of 71 Holstein cattle samples contain of 55 cows and 16 bulls extracted. PCR product were run on ABI PRISM Genetic Analyser 3130 capillary electrophoresis in the presence of GeneScan – 500 LIZ internal size standard. Fragment analysis of PCR products was then performed by GeneMapper software version 4.0. The number of all and heterozygosities calculated using GenAlex 6.4 software and polymorphic information content (PIC) and exclusion probability (PE) calculated using CERVUS 3.0 software. The panel of 12 markers showed mean PIC value 0.871 and the average of number of allele was 18.9.The mean of observed heterozigosity and expected heterozygosity shown 0.920 and 0.891 respectively. The probability of parentage exclusion (PE) obtained 0.999.

Keywords: Microsatellite Markers, Polymorphism, Heterozygosity, Polymorphic Information Content, Probability of Parentage Exclusion

INTRODUCTION

Traditionally, pedigree verification in dairy cattle has been carried out using blood groups and protein polymorphisms (Stormont, 1967). This internationally standardized system has worked well, but has some drawbacks. The number of loci used gives relatively low precision

and cannot always exclude a putative sire, even if that putative sire is not the real parent. In addition, blood typing cannot be done retrospectively, e.g., after a sire is dead. Molecular techniques using DNA-based microsatellite markers offer several advantages over traditional blood typing. Any sample from an individual (e.g., hair, milk, saliva) can be used, as long as it contains DNA; hence, the sampling technique can be noninvasive and retrospective from stored tissue or semen samples. The accuracy of the DNA test is much greater than for blood group markers, as DNA markers can have many alleles and a virtually unlimited supply of markers are available (Kappes et al., 1997).

Microsatellites are short tandem nucleotide repeats that are randomly distributed throughout eukaryotic genomes. The repeat units can range from two to six base pairs motifs (Tautz and Schlotterer, 1994). They are called also as, simple sequence repeats (SSR) (Tautz, et al.,1989), short tandem repeats (STRs), (Edwards et al., 1991) or variable number tandem repeats (VNTR). Alleles at a specific locus can differ in the number of repeats. They are generally found in nuclear genome, usually in non-coding parts of genome.

Microsatellites are "junk" DNA, and are selectively neutral (Li et al., 2002). Microsatellite loci are often hypervariable with high mutation rates and therefore are highly polymorphic in most mammalian species (Weber, 1990, Jeffreys et al., 1994).Mechanisms of mutation are believed to be unequal crossover during recombination (Smith, 1976), polymerase slippage and especially slipped-strand mispairing during replication (Levinson and Gutman, 1987) resulting in the addition or loss of one or a small number of repeats. They are inherited co dominantly in Mendelian fashion and are relatively easy to score directly.

Microsatellite markers are currently the markers of choice for molecular genetic studies such as reconstruction of phylogenetic and relationships among populations (Bowcock et al., 1994; Forbes et al., 1995; MacHugh et al., 1997), determination of paternity and kinship analyses (Glowatzki-Mullis et al., 1995; Heyen et al., 1997; Luikart et al., 1999; Schlotterer, 2004) forensic studies (Edwards et al., 1992), linkage analysis

(Francisco et al., 1996; Kappes et al., 1997; Mellersh et al., 1997), population structures (Arora and Bhatia, 2004; Bruford and Wayne, 1993). Microsatellites as DNA markers are advantageous over many other markers as they are highly polymorphic, highly abundant, co-dominant inheritance, simply to analyze and easy to score, but nevertheless this type of marker has disadvantages such as null alleles, or size homoplasy (Schlotterer, 2004).

The aim of this study was investigation of molecular characterization and polymorphism of eighteen microsatellite markers and them application for studies parentage testing and genetic diversity in Holstein cattles. Result of different parameters allele, heterozygosities, polymorphic information content (PIC) and probability of parentage exclusion (PE) will be presented.

MATERIAL AND METHOD

Molecular Markers

A common set of 18 microsatellites was selected from panel 3.1(FINNZYMES DIAGNOSTICS,F- 904,FINLAND) recommended by the International Society for Animal Genetics (ISAG)/Food and Agriculture Organization (FAO) : TGLA227 ,TGL53 , RM067, TGLA126,TGLA122,INRA23,ETH3, ETH225, BM1824, CSRM60, MGTG4B, CSSM66, BM2113, ETH10,SPS115,SPS113,BM1818, ILSTS006.Table 1 shows the characteristics of 18 markers used in the study.

Table 1: Chromosome number ,motif repeat, allele size range and fluorescent – labelled dye

Locus	Chromosome Number	Primer sequences	Allele size range(bp)	Dye
TGLA227	18	F: CGA ATT CCA AAT CTG TTA ATTTGCT R: ACA GAC AGA AAC TCA ATG AAA GC	63-115	Blue
BM2113	2	F:GCTGCCTTCTACCAAATACCC R:CTTCCTGAGAGAAGCAACACC	116-146	Blue
TGLA53	16	F: GCTTTCAGAAATAGTTTGCATTCA R: ATCTTCACATGATATTACAGCAGA	147-197	Blue
ETH10	5	F:GTTCAGGACTGGCCCTGCTAACA R:CCTCCAGCCCATTTCTCTTCTC	198-234	Blue
SPS115	15	F:AAAGTGACACAACAGCTTCTC R:AACGAGTGTCCTAGTTTGGCTGTG	240-270	Blue
SPS113	10	F:CCTCCAACACGGCTTCTCTGACTT R:CCTAACTTGCTTGAGTTATTGCCC	279-307	Blue
RM067	4	F: AAGATGTGATCCAAGAGAGAGGCA R: AGGACCAGATCGTGAAAGGCATAG	83-101	Green
TGLA126	20	F: CTA ATT TAG AAT GAG AGA GGC TTC T R: TTG GTC TCT ATT CTC TGA ATA TTC C	104-132	Green
TGLA122	21	F: CCC TCC TCC AGG TAA ATC AGC R: AAT CACATGGCAAAT AAGTACATA	133-193	Green
INRA023	3	F: GAG TAG AGC TAC AAG ATA AAC TTC R: TAA CTA CAG GGT GTT AGA TGA ACT C	194-236	Green
BM1818	23	F:AGCTGGGAATATAACCAAAGG R:AGTGCTTTCAAGGTCCATGC	248-276	Green
ETH3	19	F: GAACCTGCCTCTCCTGCATTGG R: ACTCTGCCTGTGGCCAAGTAGG	89-131	Black
ETH225	9	F: GAT CAC CTT GCC ACT ATT TCC T R: ACA TGA CAG CCA GCT GCT ACT	132-166	Black

BM1824	1	F: GAG CAA GGT GTT TTT CCA ATC R: CAT TCT CCA ACT GCT TCC TTG	170-218	Black
CSRM60	10	F: AAGATGTGATCCAAGAGAGAGGCA R: AGGACCAGATCGTGAAAGGCATAG	79-115	Red
MGTG4B	4	F: GAGCAGCTTCTTTCTTCTCATCTT R: GCTCTTGGAAGCTTATTGTATAAAG	129-153	Red
CSSM66	14	F: ACA CAA ATC CTT TCT GCC AGC TGA R: AAT TTA ATG CAC TGA GGA GCT TGG	171-209	Red
ILSTS006	7	F:TGTCTGTATTTCTGCTGTGG R:ACACGGAAGCGATCTAAACG	277-309	Red

F: Forward R: Reverse bp: Base pair

Sampling strategy

A total of 71 Holstein cattle samples were genotyped. Blood sample of 55 cows were collected from 12 different dairy herds . Semen samples of 16 bulls was obtained from The Animal Breeding Center and improve livestock production in Iran.

DNA extraction and PCR amplification

Genomic DNA of blood samples was extracted using salting – out method and DNA of semen samples extracted by extraction kit from MBST(Molecular Biotechnology System Transfer) company in Iran .The 18 microsatellite markers were amplified in multiplex polymerase chain reactions (PCRs) using fluorescence-labelled primers .PCR product were run on ABI PRISM Genetic Analyser 3130 (Applied Biosystems Inc., Foster City , CA) capillary electrophoresis in the presence of GeneScan – 500 LIZ internal size standard (Applied Biosystems Inc.,Foster City,CA), which is designed for sizing DNA fragment in the 35 – 500 bp range and provides 16 single – standard labeled fragments. The raw data were collected using Data Collection software version 4.0, which was installed on the ABI system. Fragment analysis of PCR products was then performed by GeneMapper software version 4.0 (Applied Biosystems Inc., Foster City, CA).

Statistical analysis

Allele frequencies for each locus, total number of alleles per locus (NA), estimated observed (Ho) and unbiased expected (He) heterozygosities and Shannon index were calculated using GenAlex 6.4 software .Polymorphism information content (PIC) and probability of parentage exclusion (PE) value of each locus obtained by CERVUS 3.0 software (Kalinowski et al .,2007).

$$n_e = \frac{1}{\sum pi^2}$$

P_i : frequency per alleles

$$PIC = 1 - \left(\sum_{i=1}^{k} p_i^2\right) - \sum_{i=1}^{k-1} \sum_{j=i-1}^{k} 2\, P_i^2 P_j^2$$

K: numbers of alleles

P_i and P_j : allelic frequency in i and j population

$$PE = 1 - \prod_{j=1}^{n} (1 - PE_{i})$$

PE_i : probability of exclusion in i genetic marker

RESULTS

Despite using the ABI system and its powerful performance in genotyping , some samples showed low quality in genotyping specially in BM2113, ETH10, SPS115,SPS113, BM1818 and ILSTS006 loci, thus these locus deleted. Table 2 shows the results of 12 microsatellite markers.

Table 2: Variability measures of twelve microsatellite markers in Holstein cattle

Locus	Na	Ng	Ne	H_o	H_e	I	PIC	PE
ETH3	24	71	14.70	0.958	0.932	4.15	0.921	0.950
TGLA126	17	70	9.09	0.957	0.889	3.47	0.871	0.710
ETH225	16	68	8.16	0.941	0.878	3.46	0.862	0.720
RM067	17	67	8.16	0.881	0.878	3.36	0.860	0.750
BM1824	23	71	15.38	0.915	0.935	4.22	0.924	0.845
CSSM66	18	71	8.92	0.972	0.888	3.61	0.871	0.739
INRA023	14	68	5.58	0.941	0.821	2.84	0.790	0.778
TGLA122	24	67	11.49	0.851	0.913	3.87	0.899	0.770
TGLA53	19	69	14.90	0.928	0.933	3.95	0.921	0.871
CSRM60	18	60	10.30	0867	0.903	3.63	0.887	0.782
MGTG4B	18	71	5.71	0.971	0.825	2.91	0.802	0.596
TGLA227	19	50	9.17	0.860	0.897	3.54	0.845	0.834
Mean	18.9	66.9	11.13	0.920	0.891	3.58	0.871	0.999

Na : The number of observed all, Ng : Overall number of genotypes , Ne : Effective alleles, H_o : Observed heterozygosities, H_e: Expected heterozygosities , I: Shannon Index, PIC : Polymorphism information content, PE: probability of parentage exclusion

Allele numbers, sizes and their frequencies

Number of alleles observed at a single locus ranged from 14 (INRA023) to 24 (ETH3 and TGLA122) (Table 2). The frequency distribution of alleles was in the range of 0.0014 to 0.845. The mean number of observed all was 18.9 and mean of overall number of genotypes was 66.9.

Heterozygosity

Heterozygosity is an appropriate measure of genetic variability within a population when populations are expanding (Hanslik et al., 2000). In the present study, the observed heterozygosity ranged from 0.851 (TGLA122) to 0.972 (CSSM66) where as the range for expected heterozygosity was 0.821 (INRA023) to 0.935 (BM1824) (Table 2).The average observed heterozygosity was 0.920 and the average expected heterozygosity obtained 0.891.Tenava et al,(2005) obtained the average expected heterozygosity 0.89.

Polymorphism information content (PIC)

PIC values indicate the informativeness of the microsatellite loci studied. In the present study the PIC values for all the 12 microsatellite loci ranged from 0.790 to 0.924 (Table 2). As well as, the mean Polymorphism information content (PIC) shown 0.871. All the loci showed high PIC values suggestive of more polymorphic nature. The result of PIC had similarities and differences than previous results ,for example , Stevanovic et al (2010) used 11 microsatellite markers for parentage testing in Yugoslav Pied cattle and obtained the mean of PIC 0.84 that it was similar to our result. But result of Řehout et al (2006) (0.713) and Lee et al (2006) (0.652) was different than our result.

Probability of parentage exclusion (PE)

The PE is an appropriate measure for detection a correct parent in parentage testing. This parameter calculate using CERVUS software. Figure 1 shows the samples of graph that obtained using CERVUS software. In this software , the PE value for parentage test with both parents obtained ranged from 0.596 (locusMGTG4B) to 0.95(locusETH3). All loci had PE value higher than 0.7 except MGTG4B loci .The Cumulative PE shown 0.999 .Table 1 shows the average and combined PE value for each locus for both parents. Using 11 microsatellite markers David et al. (2012) obtained the Cumulative EP 0.9901. Also, Ozkan et al (2009) and Zhang et al (2010) in investigation of parentage test showed the Cumulative PE 0.999.

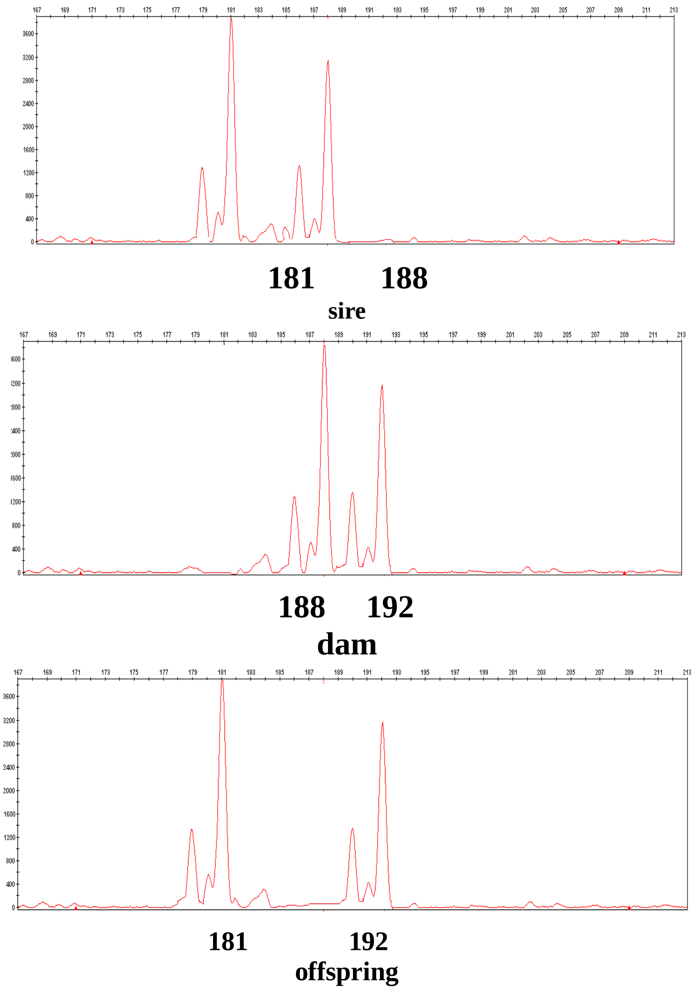

Figure 1: Samples of graphs that obtained using CERVUS software in CSSM66 loci.(This graph shows accuracy of relation between sire and offspring and between dam and offspring)

DISCUSSION

The informativeness of a polymorphic marker depends upon the number of alleles and their relative population frequencies (Taylor,1997). In this study, the mean *nA* per was 18.9 and the allele frequency of 12 microsatellite markers in 71 samples shown 201 allele. High number of alleles and PIC values were observed for ETH3, BM1824, TGLA122, TGLA53 and TGLA227. If the allele frequency of microsatellite markers be less than 0.99 or 0.95 , they are polymorphism. In this study, all of loci had the allele frequency less than 0.84, thus, all of them had high polymorphism.

The Shannon index use to calculate the level of genetic diversity. Because the highest of heterozygosity for any numbers of alleles is 1, this parameter is not accurate for calculating genetic diversity .So, Shannon index is very suitable and useful for calculating diversity of loci (Movahedin et al., 2010).In this study, the Shannon index calculated using GenAlex 6.4 software(Table 2).The Shannon index obtained range of 2.84 to 4.22 and the average Shannon shown 3.66.In our study, all of loci had the high Shannon index and the high diversity.

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 (Zhang et al. 2010).As for PE measures, the cumulative values were >0.999. So, the selected markers (12 marker) showed substantial higher cumulative PE value (0.999). These values were similar to the parentage testing power of the system developed by zhang et al. (2010) and the systems for Iranian Holstein cattles (Hashemi et al., 2012).

CONCLUSION

The polymorphism of 12 analysed markers shows that the complementary set of microsatellite DNA sequences could be used for confirming parentage in cattle, genetic diversity within and among population, molecular characterization and genetic characterization in cattle , sheep and horse.

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