Estimation of Genetic Variation in Holstein Young Bulls of Iran AI Station Using Molecular Markers

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ABSTRACT : Genetic profiles of Iranian Holstein young bulls at the national artificial insemination station were determined on the basis of individual genotypes at 13 ISAG's recommended microsatellites, the most useful markers of choice for parentage identification. In the present study a total of 119 individuals were genotyped at 13 microsatellite loci and for possible parent-offspring combinations. A high level of genetic variation was evident within the investigated individuals as assessed from various genetic diversity measures. The mean number of observed alleles per microsatellite marker was 9.15 and the number of effective alleles as usual was less than the observed values (4.03). The average observed and expected heterozygosity values were 0.612 and 0.898, respectively. The mean polymorphic information content (*PIC*) value (0.694) further reflected a high level of genetic variability. The average exclusion of probability (*PE*) of the 13 markers was 0.520, ranging from 0.389 to 0.788. The combined exclusion of probability was 0.999, when 13 microsatellite loci were used for analysis in the individual identification system. Inbreeding was calculated as the difference between observed and expected heterozygosity. Observed homozygosity was less than expected which reflects inbreeding of -3.7% indicating that there are genetic differences between bull-sires and bull-dams used to produce young bulls. The results obtained from this study demonstrate that the microsatellite DNA markers used in the present DNA typing are useful and sufficient for individual identification and parentage verification without accurate pedigree information. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 4 : 463-467)***

Key Words : Parentage Control, Genetic Diversity, Inbreeding, Microsatellite Markers

INTRODUCTION

Discoveries made by Landsteiner in the early 1900's on human blood group variability, Todd and White on blood groups in farm animals, set the scene for research into immuno-genetics and genetic variability among animals (Hines, 1999). At the present day, molecular markers that are revealing polymorphisms at the DNA level are key players in animal genetics. However, due to the existence of various molecular biology techniques to produce them, and to the various biological implications some can have, a large variety exists, from which choices will have to be made according to purposes.

In dairy cattle industry in spite of pedigree informations, blood groups and protein polymorphisms have been used for a long time to examine the genetic structure of population and establish genetic relationships between individuals respectively. As early as 1940, Irwin and co-workers at the University of Wisconsin used blood group antigens for parentage verifications in the Holstein Friesians (Hines, 1999). During the 1950's Stormont studied the blood group systems in cattle and the applications of blood groups proved to be a powerful tool in the detection of incorrect parentage, with obvious and significant implications for stud breeds (Van Marle-Köster and Nel, 2003).

However, in the last 10 years or so, the development of new genetic tools has brought about great advances in individual recognition, and DNA markers such as microsatellites have proved to be useful in clarifying population structure (Sasazaki et al., 2004; Yoon et al., 2005). They also detect population differentiation better than for example allozymes (Barker et al., 1997; Estoup et al., 1998). Microsatellite loci have gained widespread use due to their abundance in eukaryotic genomes, high polymorphism, codaminant nature, high reproducibility, and relative ease of scoring (Vignal et al., 2002). It is not uncommon to find up to 10 alleles per locus and heterozygosity values of 60% in a relatively small number of samples (Goldstein and Polack, 1997; Dorji et al., 2003). Microsatellites tend to mutate with mutation rates of up to 10^{-2} per generation (Bruford and Wayne, 1993). A large number of microsatellite markers have been mapped for various species, including humans, mice, cattle, sheep, pigs and chickens (Taylor et al., 1998; Groenen et al., 2000; Wang et al., 2004). These markers are most valuable markers in studies on genetic variability and parentage verifications.

This is the first research work on parentage verifications based on tests with genetic markers in Iran dairy industry. In the present study, we evaluated the ISAG's standards microsatellite paternity markers for estimation the degree of inbreeding and parentage control in Holstein young bulls of

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Table 1. Primer sequences used for the amplification of the microsatallite loci in Holstein young bulls of Iran AI station

Multiplex PCR	Name of locus	Fluorescent label	Concentration (nM)	Primer sequences
9-plex PCR	TGLA122	HEX	100	F: 5´-CCC TCC TCC AGG TAA ATC AGC-3´ R: 5´-AAT CAC ATG GCA AAT AAG TAC ATA-3´
	TGLA126	HEX	50	F: 5´-CTA ATT TAG AAT GAG AGA GGC TTC T-3´ R: 5´-TTG GTC TCT ATT CTC TGA ATA TTC C-3´
	TGLA227	FAM	30	F: 5´-CGA ATT CCA AAT CTG TTA ATT TGC-3´ R: 5´-ACT CTG CCT GTG GCC AAG TAG G-3´
	ETH3	TAMRA	50	F: 5´-GAA CCT GCC TCT CCT GCA TTG-3´ R: 5´-ACT CTG CCT GTG GCC AAG TAG G-3´
	ETH10	FAM	10	F: 5´-GTT CAG GAC TGG CCC TGC TAA CA-3´ R: 5´-CCT CCA GCC CAC TTT CTC TTC TC-3´
	ETH225	TAMRA	10	F: 5´-GAT CAC CTT GCC ACT ATT TCC-3´ R: 5´-ACA TGA CAG CCA GCT GCT ACT-3´
	BM2113	FAM	30	F: 5´-GCT GCC TTC TAC CAA ATA CCC-3´ R: 5´-CTT CCT GAC AGA AGC AAC ACC-3´
	BM1824	TAMRA	30	F: 5´-GAG CAA GGT GTT TTT CCA ATC-3´ R: 5´-CAT TCT CCA ACT GCT TCC TTG-3´
	SPS115	FAM	50	F: 5´-AAA GTG ACA CAA CAG CTT CTC CAG-3´ R: 5´-AAC GAG TGT CCT AGT TTG GCT GTG-3´
4-Plex PCR	SPS113	TAMRA	50	F: 5´-CCT CCA CAC AGG CTT CTC TGA CTT-3´ R: 5´-CCT AAC TTG CTT GAG TTA TTG CCC-3´
	MGTG4B	FAM	30	F: 5´-GAG CAG CTT CTT TCT TTC TCA TCT T-3´ R: 5´-GCT CTT GGA AGC TTA TTG TAT AAA G-3´
	TGLA53	HEX	40	F: 5´-GCT TTC AGA AAT AGT TTG CAT TCA-3´ R: 5´-ATC TTC ACA TGA TAT TAC AGC AGA-3´
	INRA23	FAM	30	F: 5´-GAG TAG AGC TAC AAG ATA AAC TTC-3´ R: 5´-TAA CTA CAG GGT GTT AGA TGA ACT C-3´

Iran Artificial Insemination (AI) station. The 13 recommended markers of this panel are independently segregating and highly polymorphic. All used markers have well-represented alleles and no null alleles have been reported.

MATERIALS AND METHODS

Genomic DNA isolation

Ear tissue samples were collected from 120 individuals using sample tagger developed by Biopsytec (Biopsytec GmbH, Kastanieallee, Berlin). Genomic DNA was extracted from ear tissue with the NucleoSpin Blood Quik Pure Kit (Macherey and Nagel, Düren, Germany) according to the manufacture's recommendations.

PCR multiplexes and microsatellite markers

Multiplex PCR reactions were carried out in a total volume of 20 μL, containing 10mM dNTP-mix, 2.5 mM $MgCl_2$, 2.5 U Thermo-start DNA polymerase and 100 ng DNA. The following microsatellite markers were used: TGLA227; TGLA126; TGLA122; ETH3; ETH225; ETH10; BM1824; BM2113; SPS115 in the 9-plex PCR and SPS113; MGTG4B; TGLA53; INRA23 in the 4-plex PCR. For each PCR reaction one primer was 5′-end labeled with commercially fluorescent labels TAMRA, FAM and HEX (Table 1). PCR conditions included an initial denaturation step of 15 min. at 94°C, 29 cycles of 1 min. at 94°C, 1 min. at 56°C, 1 min. at 72°C and a final extension of 10 min. at 72°C. The denatured PCR fragments were separated on ABI 377 sequencer and analyzed with Genescan software.

Data analysis

The likelihood ratio (G^2) test was used to evaluate Hardy-Weinberg equilibrium. Observed and effective number of alleles was calculated according Nei (1987). Expected heterozygosity is a useful measure of informativeness of a locus. The loci with expected heterozygosity of 0.5 or less are in general not very useful for large-scale parentage analysis. The overall expected

Table 2. Characterization of 13 microsatellite loci, showing observed number of alleles, effective number of alleles and allele's size range detected in Holstein young bulls of Iran AI station

Locus	Number of alleles *(Na)*	Effective number of alleles *(Ne)*	Allele's size range *(bp)*
BM1824	6	3.96	178-190
BM2113	6	3.38	125-139
ETH10	6	3.41	213-225
ETH225	7	3.38	140-152
ETH3	7	2.88	117-150
SPS115	7	2.52	117-260
TGLA122	12	3.94	139-260
TGLA126	8	3.09	115-149
TGLA227	11	5.30	81-149
INRA23	8	4.62	89-214
MGTG4B	11	4.49	135-210
SPS113	12	2.81	135-155
TGLA53	18	8.67	151-186
Mean	9.15	4.03	-
St. Dev	3.50	1.60	-

heterozygosity across all loci which are simply the arithmetic average of the average heterozygosities at each locus. Cervus software (version 2.0) was used to calculate allele frequencies, expected and observed heterozygosities and parentage assessment (Marshall, 2000). Polymorphic information content *(PIC)* is a measure of informativeness related to expected heterozygosity. The *PIC* value of the microsatellite loci was calculated on the basis of observed allele frequencies (Botstein et al., 1980).

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

Where P_{ij} is the frequency of the *j*th allele for the *i*th marker, and summed over *n* alleles. The effective allele number (estimates the reciprocal of homozygosity) was calculated according Hartl and Clark (1989).

$$N_e = \frac{1}{H_i} = \frac{1}{\sum_{i=1}^{n} P_i^2}$$

Where N_e is the effective allele number and H_i is the homozygosity of each locus. The exclusion probability of (*PE*) and the accuracy of the paternity assessment were estimated as described by Marshall et al. (1998) and State et al. (2000). In brief, for homozygous AA, exclusion occurs if the candidate parent is neither AA nor any of the k-1 heterozygotes AX. For heterozygous offspring AB, exclusion occurs if the candidate parent is neither AA, BB, any of the k-1 heterozygotes AX nor any of the k-1 heterozygoutes BX. The heterozygous candidate parent AB occurs both in the set of genotypes AX and the set of genotypes BX. Defining the probability of genotypes AA, AB, AX and BX as p(ii), p(ij), p(ix) and p(jx) respectively and summing across all pairwise genotypic combinations, the average probability of exclusion (*PE*) at locus E with k codominant alleles was calculated with the following model:

$$PE = 1 - \left\{ \sum_{i=1}^{k} \sum_{x=1}^{k} p(ii)p(ix) + \frac{1}{2} \sum_{i \neq j}^{k} \sum_{x=1}^{k} p(ij)p(ix) + p(jx) - p(ij) \right\}$$

The combined average probability of exclusion (*CPE*), across *n* independently inherited loci was calculated based on Marshall et al. (1998):

$$CPE = 1 - \prod_{E=1}^{n} [1 - PE]$$

In order to measure an individual's inbreeding coefficient, detailed pedigree information is required. In recent years, the developments of molecular techniques offer the opportunity to determine accurate pedigrees in populations. Such approaches have the advantages that pedigree information is not required. The Inbreeding was calculated as the difference between observed and expected heterozygosity (Lukas et al., 2002).

$$F = 1 - \left[\frac{Ho}{He} \right]$$

Where *F* is the coefficient of inbreeding, the deviation of the observed heterozygosity of an individual relative to the heterozygosity expected under random mating (Hardy-Weinberg equilibrium); *Ho* is the observed frequency of heterozygous individuals and *He* is the expected frequency of heterozygous in the population. When *F* is >0 its signifies more inbreeding than is expected at random, and when *F* is <0 its indicates that inbreeding occurred less often than would be expected at random.

RESULTS AND DISCUSSION

Table 2 summarizes the observed and effective number of alleles and size range of alleles in 13 loci. A total of 119 alleles were detected across the 13 analysed microsatellite loci. All the loci were polymorphic (≥2 alleles, Crawford et al., 1995). The analyzed loci did not show deviation from Hardy-Weinberg equilibrium, using the Markov chain method (Guo and Thompson, 1992). The number of observed alleles *(Na)* varied between six (BM1824, BM2113 and ETH10) and eighteen (TGLA53) with an average of 9.15 per locus. The effective number of alleles *(Ne)* was less than the observed values ranging from 2.52

Table 3. Statistics of genetic variation of (*Ho* = observed heterozygosity, *He* = expected heterozygosity, *PIC* = polymorphic information content) and exclusion probabilities for 13 analysed microsatellite loci in Holstein young bulls of Iran AI station

Locus	*Ho*	*He*	*PIC*	*PE*
BM1824	0.788	0.753	0.705	0.511
BM2113	0.771	0.709	0.662	0.471
ETH10	0.805	0.709	0.664	0.476
ETH225	0.720	0.711	0.663	0.471
ETH3	0.695	0.662	0.614	0.420
SPS115	0.669	0.612	0.573	0.389
TGLA122	0.797	0.756	0.718	0.546
TGLA126	0.695	0.678	0.617	0.420
TGLA227	0.856	0.818	0.793	0.646
INRA23	0.780	0.791	0.755	0.582
MGTG4B	0.757	0.782	0.750	0.585
SPS113	0.685	0.652	0.630	0.463
TGLA53	0.813	0.898	0.885	0.788
Mean	0.765	0.733	0.694	0.520
St. Dev	0.058	0.077	0.085	0.109
CPE	-	-	-	0.9997

(SPS115) to 8.67 (TGLA53) with mean of 4.03. It has been shown that increasing the number of alleles at different loci increased the mean genetic diversity in population (Moioli et al., 2001). The level of variation depicted by number of alleles at each locus serves as a measure of genetic variability having direct effect on differentiation of breeds within a species (Glowtzki-Mullis et al., 1995; Saitbekova et al., 1999). High mean number of observed alleles (allelic diversity) per locus displayed high genetic variation and indicates that the population is under mutation drift equilibrium.

Table 3 shows the statistics of genetic variation (observed and expected heterozygosity, polymorphic information content and probability of exclusion) in 13 microsatellite loci analyzed. The unbiased average expected heterozygosity (Nei, 1978) was 0.733±0.077, values ranging from 0.612 (SPS115) to 0.898 (TGLA53). The high mean heterozygosity values could be attributed to low level of inbreeding, low selection pressure and large number of alleles present in a population (Arora and Bhatia, 2004). Another parameter also indicative of the genetic variation is *PIC* estimate. The average *PIC* estimated per locus was 0.694±0.085, values ranging between 0.573 (SPS115) to 0.885 for (TGLA53). Microsatellites that have *PIC* values higher than 0.5 are considered highly informative (Botstein et al., 1980), so we can consider these loci as very informative. The lower *PIC* values than their related heterozygosities obtained in this study are in agreement with other reports (Peelman et al., 1998; Lubieniecka et al., 1999). These high estimates of *PIC* substantiated the suitability of used set of DNA markers to applications such as parentage control, linkage-mapping programs in addition, to genetic polymorphism studies in other Iranian cattle breed too. Moreover, the average *PE* of the 13 markers was 0.520±0.10, values ranging from 0.389 (SPS115) to 0.788 (TGLA53), and the combined *CPE* being 0.999. The success of paternity inference is influenced by two major features, apart from the number of candidate male and the quality of marker used. The high *CPE* values obtained from polymorphic microsattelite DNA markers at the present study indicate that these codominant molecular DNA markers can be appropriate tools in parentage control.

At the present study, the average homozygosity was 0.25±0.06, values ranging from 0.17 to 0.31. The observed homozygosity was less than expected which reflects inbreeding of -3.7% indicating that there are genetic differences between bull-sires and bull-dams used to produce young bulls. Hedrick et al. (2001) examined the predictive power of both heterozygosity and the average measure of the genetic distance between the parental gametes *(d^2)* for measuring the levels of inbreeding in a captive population of wolves of known pedigree. They found that the mean d^2 was less predictive of the known inbreeding coefficient than heterozygosity.

However, inbreeding has been the focus of considerable attention in a number of areas of biology including animal and crop production. In dairy cattle industry, success in controlling inbreeding over the long term will depend on our ability to limit genetic relationships between young bulls entering in AI progeny test programs. Consequently, we need to keep enough diversity in the population to avoid inbreeding problems in the future. On the basis of the present study it can be concluded that the microsattelite markers can be used as an appropriate tools for individual identification, parentage verification and also to infer the levels of inbreeding directly from sampled individuals.

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