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Molecular Characterization of CSN3 Alleles in Sarabi and Holstein using PCR-RFLP

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Abstract: Data on genetic differences at CSN3 (κ-casein) in bovine breeds including a DNA based typing method and the mutation responsible for variation on DNA level using the Polymerase Chain Reaction (PCR) followed by restriction enzyme digestion (to reveal a RFLP) of blood samples was collected from indigenous Iranian cattle breed Sarabi and Holstein-Friesian. The PCR products were digested with *Hind* III and then loaded onto agarose gels, stained with ethidium bromide. Gene frequencies for A and B alleles in holstein and sarabi cattle breeds were estimated 0.8284, 0.1716 and 0.7652, 0.2348, respectively. Comparison of allele frequencies indicated that there was no significant difference between observed and expected frequencies in both Sarabi and Holstein populations. The observed hetrozygosity for Sarabi and Holstein were 0.3484 and 0.2843, respectively .The estimated gene diversity from sum of squares of allele frequency, for Sarabi and Holstein were 0.3593 and 0.2843, respectively. Based on information at this locus, no evidence was found for disequilibrium in two populations. Comparison of allele frequencies of this study with those of other cattle breeds indicates that only significant differences was demonstrated between two breeds of our study with Jersey.

Key words: κ-casein, sarabi, PCR-RFLP, hetrozygosity, gene diversity

INTRODUCTION

The identification of milk protein genotypes in dairy cattle provides a unique situation in which molecular genetics may have a direct impact on quantitative traits of economic importance (Medrano and Cordova, 1990). Genetic improvement of quantitative traits is therefore relatively slow and for this reason, qualitative characters, such as polymorphisms in blood groups, milk protein types, are among those being investigated for the possibilities which they provide of improving the accuracy of estimating genetic merit of sires and cows and practicing selection at an earlier age (Lin *et al*., 1992). Several studies have reported that some of these bovine protein variants particularly certain K-CN variants, are associated with lactation performance and have a major influence on milk composition and its processing properties, including cheese yield (Marziali and Ng-Kwai-Hung, 1986; Aleandri *et al*., 1990). Specific proteins in bovine milk include four caseins (αs1, αs2, β and κ-casein), each protein showing at least two genetic variants in genus Bos (Eigel, 1984). The κ-casein variants designated A and B is known (Neelin, 1964). Variants A and B differ at amino acids 136 and 148. In 1998, Barroso applied the PCR-SSCP and PCR-RFLP method to detect K-CN variants A, B, C and E present in different European cattle breeds (Erhardt *et al*., 1989, 1993) by silver staining method (Bassam *et al*., 1991). The κ, β, αs1 and αs2 casein gene cluster distributed over a total length of approximately 200 Kb on chromosome 6 in cow and chromosome 4 in goat, sheep, man; 5 in mouse and 12 in rabbit (Eggen, 1995). The Sarabi breed of cattle represents an excellent source of biological information for studies on genetic characterization, as it results from a long process of natural selection. This breed has been kept basically with no selection for productive traits and the most important goal of breeding center was purification of this breed (homozygosity) and it should therefore maintain the genetic variability which has allowed it to adopt and survive in the adverse condition of Sarab region of Tabriz. Sarabi breed is the most interesting Iranian indigenous cattle (Bos Taurus) not only because of its economic importance to the Sarab region of Tabriz-Iran, but also its purity. This study was performed to investigate allelic diagnosis of κ-casein locus in Sarabi cattle and its comparison with Holstein.

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MATERIALS AND METHODS

Samples: Blood samples were supplied from Holstein flocks at the Sheikh Hasan cattle breeding center and some of animal farm in East Azarbaijan and from Sarabi flocks at the Shabestar and Sarab cattle breeding center, then brought to Molecular Breeding Lab, Faculty of Agriculture, University of Tabriz, Tabriz, Iran. All samples were kept on -70°C until DNA extraction. In order to optimization of DNA extraction and PCR condition 10 sample of Holstein cattle from domestic farm of Agriculture faculty were supplied.

DNA Extraction: DNA was extracted within 24 h from 450 μl peripheral blood samples of 102 unrelated Holstein- Frisian cattle breed and 66 Sarabi cattle breed from mentioned flocks, containing 0.1 volume EDTA (ethylene diaminetetracetic acid) as an anticoagulation agent according to boiling method as described by Sambrook (2001).

DNA amplification by PCR: A 453 bp fragment containing exon IV of the bovine CSN3 gene was amplified by polymerase chain reaction (PCR), using a Gene Amp PCR system (Biometra) and primers corresponding to the same region in cattle (Barroso, 1998; Gen Bank: X14908). These primers were primarily analyzed using Oligo Explorer-5 software by the Gen Bank: X14908 and cDNA sequence of bovine CSN3 gene (Alexander *et al*., 1988). The PCR reaction was performed in 25 ml, using a PCR kits (Pazhoohesh Azma kh) with lyophilized component. Each tube contained 1U *Taq*-Polymerase, 200 μM dNTP, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH = 9), 50 mM of KCl. To this mixture 1 μl of each primers (20 pmol) include forward (5 μL TGT GCT GAG TAG GTA TCC TAG TTA TGG) and reveres (5′GCG TTG TCT TCT TTG ATG TCT CCT TAG) from TIB MOLBIOL Germany, 5 μl of DNA (20 ng/μl) and 1 drop of mineral oil were added. The amplification began with denaturation at 94°C for 5 min, followed by 34 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 2 min with a final extension step of 72°C for 5 min.

Gel documentation: PCR products then separated electrophoretically in agarose (GibCo.BRL) gels in TAE buffer with 100 bp DNA Ladder. Staining is carried out with ethidium bromid and gel documentation was done with UV tranilluminator of Biodoc Analysis system.

PCR-RFLP: Ten microliter of the PCR products were digested with 5U, HindIII enzyme. Samples were incubated at 37°C for 3 h. The visualization of PCR fragments and digestion products was performed in 1.6% Ethidium bromide stained agarose gels following standard procedures (Sambrook, 2001).

Diversity: The study of evolution is that of characterizing the extent and causes of genetic variation. The simplest descriptors are just the frequencies of alleles or genotypes, but emphasis in this study will be given to hetrozygosity and gene diversity.

The frequency of hetrozygotes is important, since each hetrozygote carries different alleles and represent the existence of variation.

Hetrozygosity: Hetrozygosity, which is a simple measure of genetic variation in a population, is the amount of hetrozygosity observed and this is reported for a single locus in this study. If n_{luv} is the observed count of A_uA_v hetrozygotes, $u \neq v$, at locus *l* of a sample of size n, then the sample herozygote frequency at this locus is (Weir, 1996):

$$H = \sum_{u} \sum_{u \neq v} n_{luv} / n$$

Gene diversity: An alternative measure of variation, often referred to loosely as average hetrozygosity, but more properly known as gene diversity, is formed from the sum of squares of allele frequencies sampling properties of gene diversity will develop on the genotype as well as the allele frequencies. If P_{lu} Is the frequency of the *uth* allele at the *lth* locus, the gene diversity at this locus is (Weir, 1996):

$$D_l = 1 - \sum_{u} P_{lu}^{2}$$

RESULTS

DNA amplification: Figure 1 shows the result of a PCR amplification of κ-casein Exon IV that is consist of 10944 polymorphic sites along with 50 bp DNA ladder in two sides of the gel. The pair of primers used for κ-casein were designed to amplify the region between nucleotides 10594 and 11048 of Exon IV Gen Bank: X14908 (Barroso, 1998) as their lengths correspond to those expected according to the position of the primers deduced from the described nucleotide sequence of the corresponding genes from Bos-Taurus cattle. These primers allowed amplification of the fragment with expected length of 453 bp.

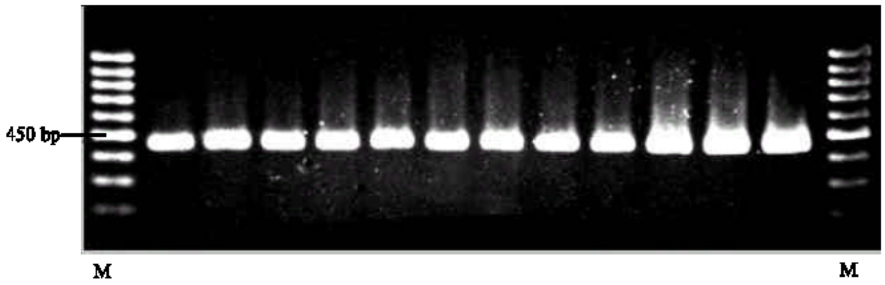

Fig. 1: PCR products analyzed by electrophoresis in a 1.6% agarose gel with ethidium bromide staining. M (Molecular weight marker: 1031-80 bp) (Fermentase#smo241)

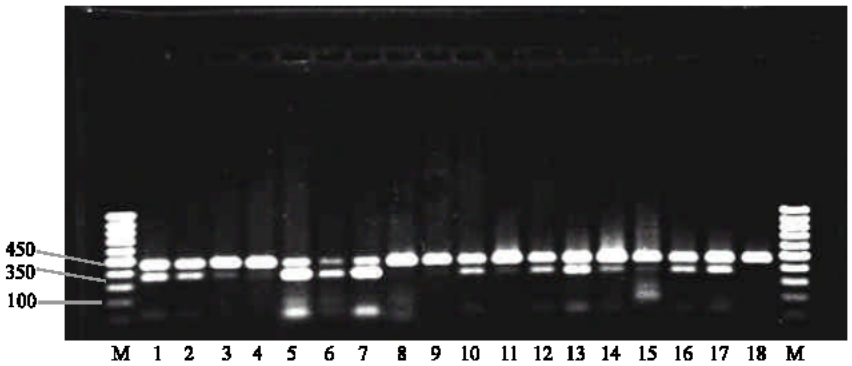

Fig. 2: Restriction fragment pattern of κ-casein after digestion with Hind III Lanes 1, 2, 5, 7, 10, 12, 13, 16, 17: genotype AB., 3, 4, 8, 9, 11, 14, 15, 18: genotype AA, lane 6: genotype BB, M (Molecular weight marker: 1031-80 bp) (Fermentase#smo241)

Table 1: Sizes of fragments for each genotype resulting from digestion of the PCR Products with restriction enzyme *Hind* III

Genotype	Restriction enzyme-*Hind* III fragments		
AA	453	-	-
AB	453	354	99
BB	-	354	99

PCR-RFLP: All samples were evaluated with PCR-RFLP technique using *Hind* III restriction enzyme. The use of PCR-RFLP to detect new mutation is only feasible if such mutation creates or destroys a restriction target for the enzyme used. Table 1 shows the expected sizes of the bands with *Hind* III restriction enzyme. The *Hind* III misidentified allele E, however it was possible to differentiate allele B from A as allele A was the one that remained undigested. Figure 2 shows the restriction patterns of alleles A and B after digestion, while the 99 bp bands were not distinguishable in agarose gels. Identification of A and B alleles of κ-casein was performed by amplification of DNA fragment of 453 bp located across exon IV, by the PCR-RFLP method. One *Hind* III restriction site is found in position 354 of the amplified fragment of allele B. So DNA fragment amplified from allele B shows only one restriction site, resulting in two fragments of 354 and 99 bp. No site is found for *Hind* III in the allele A and therefore allele A was characterized by the absence of *Hind* III recognition site. Thus restriction digests of allele A yielded 453 bp band, while 99 and 354 bp fragments in the case of B allele. This leads to a differentiation between samples with κ-casein genotypes AA, AB and BB using *Hind* III where, the bands were easily distinguished by agarose gel electrophoresis and ethidium bromid staining but, the 99 bp band was not clear in agarose gel (Fig. 2) and acrylamid gel has to be used for detection of this band. The technology described here can be applied to differentiate genes with allele that differs at endonuclease restriction sites, including genes involved in control of other quantitative variation. Pinder *et al* (1991) reported that restriction fragments of *Hind* III digests of amplified DNA with different κ-casein genotypes can be identified in agarose and polyacrylamid gels.

Gene frequency and diversity: Results showed that genotype frequencies of Sarabi and Holstein were 0.59091, 0.34848, 0.6061 and0.6863, 0.2843, 0.0294 for AA, AB and BB, respectively. Frequency of alleles A and B estimated from genotype frequencies for Sarabi were 0.7652±0.0361, 0.2348±0.0361 while 0.8284±0.0265, 0.1716±0.0265 in the

Table 2: Allele frequencies at the κ-casein locus in six bovine breeds

	κ-Casein Alleles		
Breeds	A	B	Reeferenc
Sarabi	0.762	0.238	Present study
Holstein	0.827	0.173	Present study
Jersey	0.112	0.888	Tambasco (1998)
Pantanerio	0.762	0.218	Lara (2002)
Polish Holstein	0.770	0.230	Strzalkoska, 2002
Pinzguaer	0.783	0.211	Erhardt(1993, 1989)

case of Holstein. The analysis of variance for two populations based on hetrozygosity showed that there is no significant difference between these populations, statistically.

Based on information at this locus, no evidence of disequilibrium was found in two populations. In other words deviation of observed from the expected genotype frequencies under Hardy-Weinberg equilibrium were not significant ($p<0.01$), suggesting that the Sarabi and Holstein cattle population are in equilibrium for κ-casein locus, i.e., there is no selection advantage for any of the genotypes (Table 2).

The observed hetrozygosity for Sarabi and Holstein were 03484 and 0.2843, respectively .The estimated gene diversity from sum of squares of allele frequency represented by Weir (1996), for Sarabi and Holstein were 0.3593 and 0.2843, respectively.

Comparison of allele frequencies in κ-casein locus between Sarabi and Holstein populations in this study showed no significant differences, statistically. When allele frequencies at this locus for Sarabi and Holstein are compared with those in other breeds studied by others (Table 2), only significant difference was found between populations in this study with Jersey and Brown Swiss.

DISCUSSION

Although it is currently accepted that genetic variability in bovine landraces is high but, genetic variation at the κ-casein locus has not been previously reported for Sarabi cattle and observed hetrozygosity in our study was indicated a high genetic variability in this native population. Goodness of fit test between observed and expected genotype frequencies under Hardy-Weinberg equilibrium were not significant ($p \leq 0.05$), suggesting that the Sarabi and Holstein cattle population samples are in equilibrium for the κ-casein locus, i.e., there is no selective advantage for any of the genotypes. Genetic characterization of cattle breeds has been demonstrated that the B allele of κ-casein occurs at higher frequencies in breeds originating from Bos Taurus than of those of Bos Indicus origin (Golijow *et al*., 1996). The fact that the κ-casein B allele was detected at a high average frequency in Podolic, Italian and Iberian breeds suggesting that κ-casein B allele may be present in the common ancestors of the Bos and Bison genera. The When results for Sarabi are compared with those in other breeds (Table 2), it is apparent that allele frequencies in Sarabi are closed to those observed for breeds of Bos Taurus and Bos Indicus origin. But there are many differences between Sarabi and Jersey in gene frequencies of kappa casein locus. Tambasco (1998) reported a gene frequency of 0.88 for the B allele of κ-casein in Jersey cattle, which is in agreement with the cheese making properties attributed to milk produced by this breed, given is high protein content and the curd firmness obtained. According to Marziali and Ng-Kwai-Hang (1986), cheese production can be increased by 10% if milk is from cows of the BB genotype for κ-casein, when compare with milk from AA animals, therefore Sarabi breed can be useful in cheese making and in practical all cheese from this cow has best quality. Milk composition, namely its protein content and yield, are the main factors influencing the yield of cheese production. The polymorphisms of milk proteins affect the milk composition and cheese quality. Caseins, in popular, have been proposed as polymorphic markers for the selection of dams and sirs in order to improve the yield quality of cheese and in particular, have been proposed as polymorphic marker for the selection of cattle in order to improve the yield and the quality of cheese. The PCR-RFLP analysis of milk protein genes could be valuable alternative approach for establishing allelic variants useful as marker to aid selection .The genotype of cattle at the κ-casein locus can be ascertained using PCR-RFLP technique. This typing is rapid and can be performed using easily available source of DNA, i.e., Blood and semen and is thus effective for samples of any age of sex. By means of selection based on parent genotypes at the DNA level it will be possible to obtain cattle population producing milk. Most investigation was showed that the frequency of B allele of kappa casein in Holstein and native cattle breeds is low. Our results provide evidence that there is a high variability within Iranian indigenous cattle breed "Sarabi" for the κ-casein locus, with a frequency of B allele of 0.2348. Using information about genomic organization at the κ-casein locus (Alexander *et al*., 1988) it was possible to design two primers and to amplify a target sequence in which the allele specific mutations of κ-casein A and B are located. Fragment obtained by direct restriction analysis of the amplification product after *Hind* II and *Hinf* I digest are corresponding to the results of Medrano and Cordova (1990) concerning κ-casein AA, AB and BB. Further it could be shown that restriction analysis with *Hind* III, *Taq* I (Leveziel *et al*., 1988), Hinf I (Medrano and Cordova), involves the risk of misidentification as κ-casein A and κ-casein E give

identical fragments. The primers described earlier by Denicourt *et al.* (1990), Medrano and Cordova (1990) and Pinder *et al.* (1991), are not suitable for the detection of other κ-casein alleles such as CSN3 f allele as they do not include the polymorphic *Hha* I site.

Therefore it is necessary to use another enzyme or other technique such as PCR-SSCP in order to identification EE ones. As the AA are may be EE in samples and *Hind* III couldn't detect it, so it is necessary to use enzymes *Hinf* I, *Hind* III, *Hae* III in order to differentiate genotypes AA, BB, EE and so on.

PCR amplification and RFLP analysis is a powerful and simple method for allelic diagnosis. The specificity of PCR makes it possible to amplify segments of the genome containing allelic variations that differ at restriction enzymes sites and to identify the alleles by digestion of the amplified samples. The large amount of DNA generates by PCR make it possible to identify the variants without use of labeled probes. Since DNA can be isolated from any tissue including sperm, the method described here can be applied to breeding programs that rely on artificial insemination. The use of PCR-RFLP to detect new mutation is only feasible if such mutations create or destroy a restriction target for the enzyme used. In other words PCR-RFLP technique allows CSN3 typing and offers the possibility for the identification of the complete casein cluster or any other genes at DNA level. In beef cattle, maternal ability is a major factor affecting production efficiency, especially under extensive systems. As milk production is an important component of maternal ability, it would be important to evaluate in beef breeds, particularly those kept in tropical condition, if a relationship exists between a cow's genotype at the κ-casein locus and her maternal performance, as well as possible association with other productive and reproductive traits. If that is the case, selection response in beef production systems could be enhanced by including genotypic information in selection decision.

CONCLUSIONS

A Sarabi cattle shows a high degree of genetic variability for the κ-casein locus, with a frequency of the B allele of 0.238. As this allele has been shown to be favorably related to milk composition in dairy cattle breeds, it can be anticipated that an association may also exist with maternal performance in beef breeds. Therefore studies aimed at establishing this possible relationship are of crucial importance, as selection could be enhance by the inclusion of genetic markers in selection decision.

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REFERENCES

Aleandri, R., L.G. Bultazzoni, J.C. Schneider, A. Caroli and A.R. Davoli, 1990. The effect of milk protein polymorphism on milk components and cheese-producing ability. J. Dairy Sci., 73: 241-255.

Alexander, J., A.F. Stwart, A.G. Mackinlay, T.V. Kapelineskaya, T.M. Tkach and S.I. Gorodetsky, 1988. Isolation and characterization of the bovine k-casein gene. Eur. J. Biochem., 178: 395-401.

Barroso, A., S. Dunner and J. Canon, 1998. Detection of bovine kappa-casein variants A, B, C and E by means of polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). J. Anim. Sci., 76: 1535-1538.

Bassam, B.J., G. Catano-Anolles and D.M. Gresshoff, 1991. Fast and sensitive silver attaining of DNA in polyacrylamide gel. Anal. Biochem., 196: 80-83.

Denicourt, D., M.D. Sbour and A.J. Mc Allister, 1990. Detection of bovine k-casein genomic variants by the polymerase chain reaction method. Animal Genet., 21: 215-216.

Eggen, K.A. and R. Fries, 1995. An integrated cytogenetic and meitic map of the bovine genome. Animal Genetics, 26: 215-238.

Eigel, W.N., J.E. Butler, C.A. Crnstrom, H.M. Jr. Farrell, V.A. Harwalker, R. Jenness and R. Mcl. Whitney, 1984. Research papers: Nomenclature of proteins of cow's milk, Fifth Revision. J. Dairy. Sci., 67: 1599-1631.

Erhardt, G., 1989. K-caseins in bovine milks. Evidence for a further allele (k-CnE) in different breeds. J. Animal Breed. Genet., 106: 225-231.

Erhardt, G., 1993. Allele frequencies of milk proteins in German cattle breeds and demonstration of As 2-casein variants by isoelectric focusing. Arch. Tierz. Dummerstorf, 36: 145-152.

Golijow, C.D., G. Giovambattista, M. Poli, F.N. Dulout and M.M. Lojo, 1996. κ-casein genefrequencies support subdivision and historicalorigin of Argentine Creole cattle. Braz. J. Genet., 19: 583-586.

Leveziel, H., L. Metenier, M.F. Mahe, J. Chopain, J.-P. Furet, G. Paboeuf, J.C. Mercier and F. Grosclaude, 1998. Identification of two common alleles of the bovine-casein locus bye the RFLP technique, using the enzyme Hind III. Genetique Election Evalution, 20: 247-254.

Lin, C.Y., M.P. Sabour and A.J. Lee, 1992. Direct typing to milk proteins as an aid for genetic improvement of dairy bulls and cows: A Review Animal Breed. Abst., 60: 1-10.

Marziali, A.S and K.F. Ng-Kwai-Hung, 1986. Relationships between milk protein polymorphisms and cheese yielding capacity. J. Dairy. Sci., 69: 1193-1201.

Medrano, J.F. and E.A. Cordova, 1990. Genotyping of bovine kappa casein loci following DNA sequence amplification. Biol. Technology, 8: 144-146.

Neelin, J.M., 1964. Variants of κ-casein revealed by improved starch gel electrophoresis. J. Dairy. Sci, 47: 506-509.

Pinder, S.J., B.N. Perry, C.J. Skidmore and D. Savva, 1991. Analysis of polymorphism in the bovine casein genes by use of the polymerase chain reaction. Animal Gene., 22: 11-20.

Sambrook, J., 2001. Molecular Cloning: A Laboratory Manual (3-Volume Set), 3rd Edn. Cold Spring Harbor Laboratory. Chap 6.

Schleiben, P. and Rotmann, 1992. Identification of bovine kappa-casein C by using the polymerase chain reaction. J. Animal Breed. Genet., 190: 153-155.

Strzalkowska, N., J. Krzyzewski, L. Zwierzchwski, and Z. Ryniewier, 2002. Effect of k-casein and B-lactoglobulin loci polymorphism, cow age, stage of lactation and somatic cell count on daily milk yield and milk composition in polish black and with cattle. Anim. Sci. Papers and Reports, 20: 21-35.

Tambasco, M.D., 1998. Deteção de polimorfismodos genes de K-casína e Beta-lactoglo-bulina em animais da raça Jersey. Monografia:Universidade Federal de São Carlos, SP.

Weir, S. Bruce, 1996. Genetic Data Analysis II: Methods for Discrete Population Genetic Data 2nd (Edn.). Sinauer Assoc., Sunderland, Massachusetts. Chap: 4