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Genetic polymorphism of milk protein variants and their association studies with milk yield in Sahiwal cattle

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The objective of this study was to determine the allele and genotype frequencies of genetic variants in five milk protein genes and estimate the effect of these variants on milk yield in Sahiwal cattle. Genotypes of five milk protein genes (*alpha s1 casein, beta casein, kappa casein, alpha lactalbumin and beta lactoglobulin*) were detected using SNaPshot genotyping method. All the five milk protein genes studied exhibited polymorphism with high allele frequencies of 0.51 for *alpha s1casein C*, 0.93 for *beta casein A2*, 0.92 for *kappa casein A*, 0.93 for *alpha lactalbumin B* and 0.91 for *beta lactoglobulin B*. Statistically significant differences (p > 0.05) were observed in *kappa casein* genotypes AA (AA) and AB (AC) that is, genotype AB had more milk yield in 1st lactation (422 kg) and 2nd lactation (612 kg), respectively. In conclusion, the AB genotype identified in *kappa casein* gene is associated with higher milk production therefore incorporation of AB and BB genotypes for *kappa casein* may help to improve the milk yield in Sahiwal cattle population of Pakistan. To the best of our knowledge, this is the first detailed study involving frequency distribution of genetic variants and their effects on milk yield in *Bos indicus* Sahiwal cattle of Pakistan.

Key words: Genetic variant, milk protein genes, Sahiwal cattle.

INTRODUCTION

For centuries, animal breeders have greatly and effectively manipulated the genomes of livestock species and enhanced production traits in their herds by selecting superior individuals as predecessors for the next genera-tions. This indirect use of hereditary information without involving molecular knowledge may give useful results in terms of production but may ignore some reproductive traits of animal. Hence, there is need to use selection methods that are based on genomic studies (André, 2012). Genes that are correlated with performance parameters can improve the estimation of breeding value and hence can work as a suitable supplement to conventional breeding procedures. Genetic polymorphism related to the differences in animal performance can be taken into account in the selection process.

Milk is an important source of essential nutrients for lactating calves and a key raw material for human food preparations (Reinhardt et al., 2012). All over the world people fulfill approximately 13% of their protein requirement from milk and milk products. Bovine milk proteins are generally classified as caseins, which make up about 80% of the milk proteins, consisting of four proteins: Alpha S1 (CSN1S1, 39-46% of total caseins), alpha S2 (CSN1S2, 8-11%), beta (CSN2, 25-35%), and kappa (CSN3, 8-

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Abbreviations: NCBI, National Centre of Biotechnology Information; **PCR,** polymerase chain reaction; **SNP,** single nucleotide polymorphism; **MFGM,** milk fat globule membrane; **RFLP,** restriction fragment length polymorphism; **SSCP,** single strand conformation polymorphism.

15%) (Eigel et al., 1984). Whey proteins make about 16% of the total milk protein and contain two major proteins *alpha lactalbumin* and *beta lactoglobulin*. Other minor part is made by peptones/low molecular weight peptides (3%) and milk fat globule membrane (MFGM) proteins (1%) (D'Alessandro et al., 2011).

Ruminant's milk proteins are coded by highly polymorphic genes, containing an unusually large number of polymorphisms (Nilsen et al., 2009). Since the pioneer work done by Aschaffenburg and Drewry in 1955 who discovered alleles A and B of β lactoglobulin in cattle the researchers worldwide became interested in genetic polymorphisms of major milk proteins. This chain of studies continued and according to a recent review by Caroli et al. (2009) of milk protein variants, 9 αs1 - CN (A, B, C, D, E, F, G, H, I), 4 αs2 -CN (A, B, C, D), 12 β-CN (A1, A2, A3, B, C, D, E, F, G, H1, H2, I), 14 κ-CN (A, A1, B, B2, C, D, E, F1, F2, G1, G2, H, I, J), 11 β-LG (A, B, C, D, E, F, G, H, I, J, W), and 3 α-LA (A, B, C), modified from Farrell et al. (2004) have been reported.

These allelic forms are controlled by codominant autosomal genes according to the Mendelian mode of inheritance. The different genetic variants of milk proteins differ from each other by only a few amino acid substitutions or deletions within the polypeptide chain (Eigel et al., 1984). Several studies have been carried out to determine the frequencies of genetic variants of milk proteins in different cattle breeds (Erhardt, 1996; Baranyi et al., 1996; Lien et al., 1999; Jeichitra et al., 2003; Caroli et al., 2004) and possible relationships between milk protein polymorphism and economically important production traits, milk com-position, and quality have been widely studied (Yasemin and Cengiz, 2006) due to the potential use of milk protein types as an aid to genetic selection. However no compre-hensive and detailed studies are available in Sahiwal cattle breed. The study of various allelic forms is impor-tant in terms of their effect and frequencies in this breed.

The Sahiwal is breed of *Bos indicus*, known to have the greatest potential for milk production, growth and reproductive efficiency in tropical environments compared with other *B. indicus* breeds (Mwandotto, 1994; Dahlin et al., 1998; Khan et al., 1999; Muhuyi et al., 1999). In Pakistan, the major breeding tracts of Sahiwal lie in Montgomery (Sahiwal) district Rachagani et al. (2006). Its ability to endure and produce in harsh environments coupled with its dual purpose role has widened the distri-bution of this breed in tropical and subtropical countries. Among *B. indicus* breeds, the Sahiwal is the most frequently used in dairy crossbreeding in the tropics (Muhuyi, 1997; Kahi et al., 2000).

Milk protein genetic variability in cattle has been extensively studied at both the DNA and protein levels for evolutionary and biodiversity analyses (Caroli et al., 2004). Typing milk proteins at the DNA level does not require the gene product, which renders feasible the genotyping of males and non-lactating females and even embryos.

Recent advances in single nucleotide polymorphism (SNP) detection allow different genotyping ways of detecting poly-merphisms. One of these techniques, SNaPshot geno-typing has been optimized to genotype SNPs in the bovine *αS1-casein*, *β-casein*, *κ-casein*, *α-lactalbumin* and *β-lactoglobulin* genes. SNaPshot genotyping is a newly optimized rapid and efficient screening procedure that could provide more accurate predictions of breeding values of animals to be selected, and thus improve response to selection.

This study was designed to identify variants in five milk protein genes, three caseins (αS1, β and κ) and two whey proteins (*α-lactalbumin* and *β-lactoglobulin*) and to determine allele and genotype frequencies of milk protein genetic variants in Sahiwal cattle of Pakistan. We also investigated the association between observed milk protein genetic variants and milk yield in this group of animals. Moreover, we report the effects of identified milk protein genetic variants on milk yield. Although polymorphism was observed in genes of all five studied proteins, only *Kappa casein* polymorphism had statistically significant ($p > 0.05$) association with milk yield in Sahiwal cattle. Genotype AB of *Kappa casein* had more milk yield in 1st lactation (422 kg) and 2nd lactation (612 kg), respectively. Hence genotype information at an early age can help the farmers to improve milk yield by replacing tradition selection methods currently being used in Pakistan.

MATERIALS AND METHODS

Phenotypic data and genomic DNA samples

Phenotypic data of the selected animals was collected which include animal's age, milking record, calving record, sire and dam names. Blood samples of 120 Sahiwal cattle breed were collected from Research Centre for Conservation of Sahiwal cattle (RCCSC, Khanewal) and Livestock Production Research Institute (LPRI, Okara). DNA was extracted using a standard protocol (Sambrook et al., 2001).

Primers design

Primers were designed using the bovine αs1casein, β casein, *kappa casein*, *α-lactalbumin* and *β-lactoglobulin* gene sequences available at the National Centre of Biotechnology Information (NCBI) website. Promoter regions of α-s1 casein and *α-lactalbumin* were amplified by polymerase chain reaction (PCR) using primers AS1CN and PLAL while exon VII of *beta casein*, exon IV of *kappa casein a*nd exon IV of *beta lactoglobulin* were amplified by PCR using primers X7.1Bcn, KCN and X4Blg, respectively (Table 1). Primers for SNaPshot SNP genotyping were designed using the website http://www.basic.northwestern.edu/biotools/oligocalc.html. The primers were tailored to immediately end 5' site before the target SNP (Table 1).

Genomic DNA amplification

PCR reaction (25 µl total volume) included 100 ng of bovine genomic DNA, 0.16 pmol of amplification primer (Table 1), 2.5 U *Taq* DNA polymerase, 2 mM $MgCl_2$ and 100 uM dNTPs. Thermal profile included initial denaturation for 3 min at 94°C, followed by 35

Table 1. Primer sequences.

Marker	Primer sequences (5'-3')	Tm (°C)	Amplicon size
AS1CN (a)	F-TGCATGTTCTCATAATAACC R-GAAGAAGCAGCAAGCTGG	52	310
7.1BCN (b)	F-GATTTGTTTTCCTTCTTTCCAGGAT R-GTTGGAGGAAGAGGCTGGTG	58	357
KCN (c)	F-ATCATTTATGGCCATTCCACCAAAG R-GCCCATTTCGCCTTCTCTGTAACAGA	60	350
PLAL (d)	F-AGATTCTGGGGAGGAAAGGA R-GGGTGGCATGGAATAGGAT	58	250
X4BLG (e)	F-TGGGGGCTGACCAGAAAC R-CTCCTCCTCCTGGGAATCAA	60	282
AS1CN (f)	Forward 1st round TGCATGTTCTCATAATAACC Reverse 1st round GAAGAAGCAGCAAGCTGG Extension Primer sequence GAGAGTTTACAACAAAGAAG	56	310 21
BCN (g)	Forward 1st round AACATCCCTCCTCTTACTCAAACCCCT Reverse 1st round ATATCTCTCTGGGGATAGGGCACTGCT Extension Primer sequence GTTGAGCCCTTTACTGAAA	66	338 20
KCN (h)	Forward 1st round AACATCCCTCCTCTTACTCAAACCCCT Reverse 1st round ATATCTCTCTGGGGATAGGGCACTGCT Extension Primer sequence GTTGAGCCCTTTACTGAAA	60	350 20
ALAL (i)	Forward 1st round AGATTCTGGGGAGGAAAGGA Reverse 1st round GGGTGGCATGGAATAGGAT Extension Primer sequence GGGGGTCACCAAAATGAT	58	250 19
BLG (j)	Forward 1st round Primer TGGGGGCTGACCAGAAAC Reverse 1st round Primer CTCCTCCTCCTGGGAATCAA Extension Primer sequence AGCCCGAGCAAAGCCTGG	60	282 19

a, Sequencing primers for promoter region of *alpha s1 casein* gene; b, sequencing primers for exon 7 of *beta casein* gene; c, sequencing primers for exon 4 of *kappa casein* gene; d, sequencing primers for promoter region of *alpha lactalbumin* gene; e, sequencing primers for exon 4 of beta lactalbumin gene; f, SNaPshot primer set for *alpha s1 casein* gene; g, SNaPshot primer set for *beta casein* gene; h, SNaPshot primer set for *kappa casein*; I, SNaPshot primer set for *alpha lactalbumin* gene; j, SNaPshot primer set for *beta lactoglobulin*, bp: base pai; Tm, melting temperature.

cycles of 45 s at 94°C, 45 s at 56°C, 45 s at 72°C and final extension for 10 min at 72°C using a GeneAmp PCR System 9700 (Applied Biosystems, CA). Annealing temperatures for studied milk proteins are also mentioned in Table 1.

PCR amplification for sequencing

The PCR products from 120 cattle DNA samples were purified by Microcon (Millipore Corporation, MA) and sequenced using the Big Dye Terminator Cycle Sequencing v3.1 Ready Reaction kit on an ABI PRISM 3730 automated sequencer (Applied Biosystems, CA).

PCR amplification for SNaPshot genotyping

The SNaPshot® Multiplex Kit a single-tube was used to interrogate SNPs at known locations using recommended protocol from Applied Biosystems, CA.

Genotyping by SNaPshot protocol

The minisequencing reaction produced one (homozygote) or two (heterozygote) peaks depending on the genotype at this *locus*. Three genotypes each for *alpha s1 casein*, *kappa casein* and *beta lactoglobulin* were studied in 120 animals, while beta *casein* and

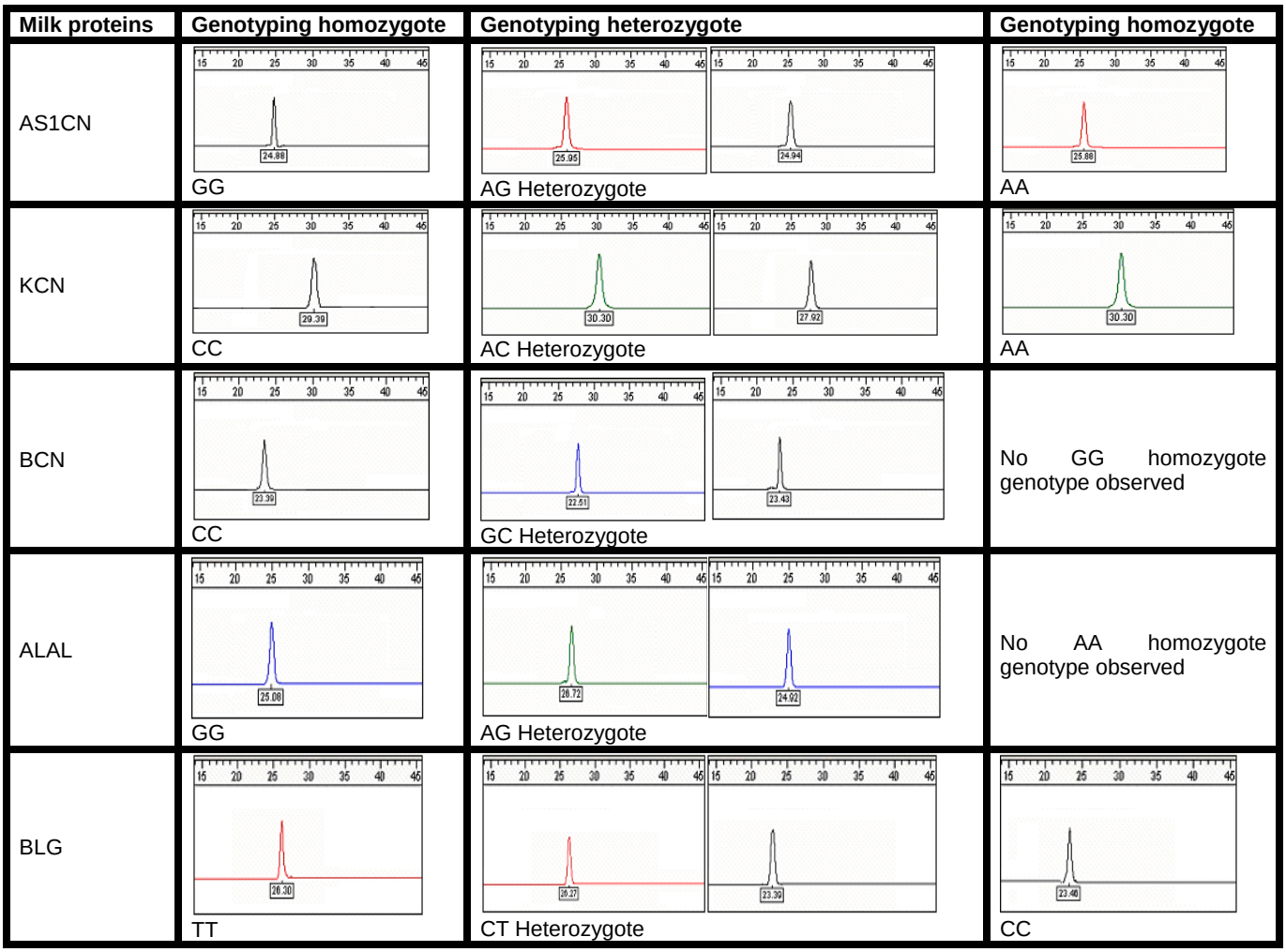

Figure 1. SNaPshot genotyping. AS1CN, *alpha s1 casein*; KCN, *Kappa casein*; BCN, *beta casein*; ALAL, *alpha lactalbumin*, BLG, *beta lactoglobulin*.

alpha lactalbumin showed only two genotypes. For the mini-sequencing technique, black color is assigned to ddCTP and blue color is assigned to ddGTP which is opposite to sequencing where black color depicts ddGTP and blue stands for ddCTP. Forward extension primers were used for *beta casein*, *kappa casein*, *alpha lactalbumin* and *beta lactoglobulin* whereas reverse extension primer was used to genotype *alpha s1 casein* reported variant. As a result, red color peak for A allele (B variant) and black color peak for G allele (C variant) were observed (Figure 1).

Statistical data analysis

Gene counting method (Chang, 1995).was used to estimate allele and genotypic frequencies of the five milk proteins. The chi-square test was used to find association of different milk protein genotypes with milk production. Influences of milk protein loci on milk yield were analyzed by linear model without interaction as follows:

$$Yijkl = \mu+Pi+Gi+eijkl$$

Where, Yijkl, is the Observed value of milk yield; μ, is the population mean; Pi, is the fixed effect of parity; is the Gi, fixed effect of milk protein genotypes (i = 1, 2….); and eijkl, is the random residual effect.

All data were input by Excel and Statistical Program for Social Sciences (SPSS Version 15.0) was used to adjust 1st lactation and 2nd lactation milk records obtained from dairy farms, those greater than 150 and less than 305 days were fixed to 305 days (simple linear regression model) and one way Analysis of Variance for statistical significance of genotypes.

RESULTS AND DISCUSSION

Allele and genotype frequency distribution

The SNaPshot protocol was successfully used to genotype all the alleles of five milk protein genes. Genotyping one hundred and twenty animals revealed ten DNA polymorphisms (alleles) in five milk protein genes and their allele frequencies were also calculated. Figures 2 and 3 summarize the estimated genotype and allele frequencies of the *alpha*

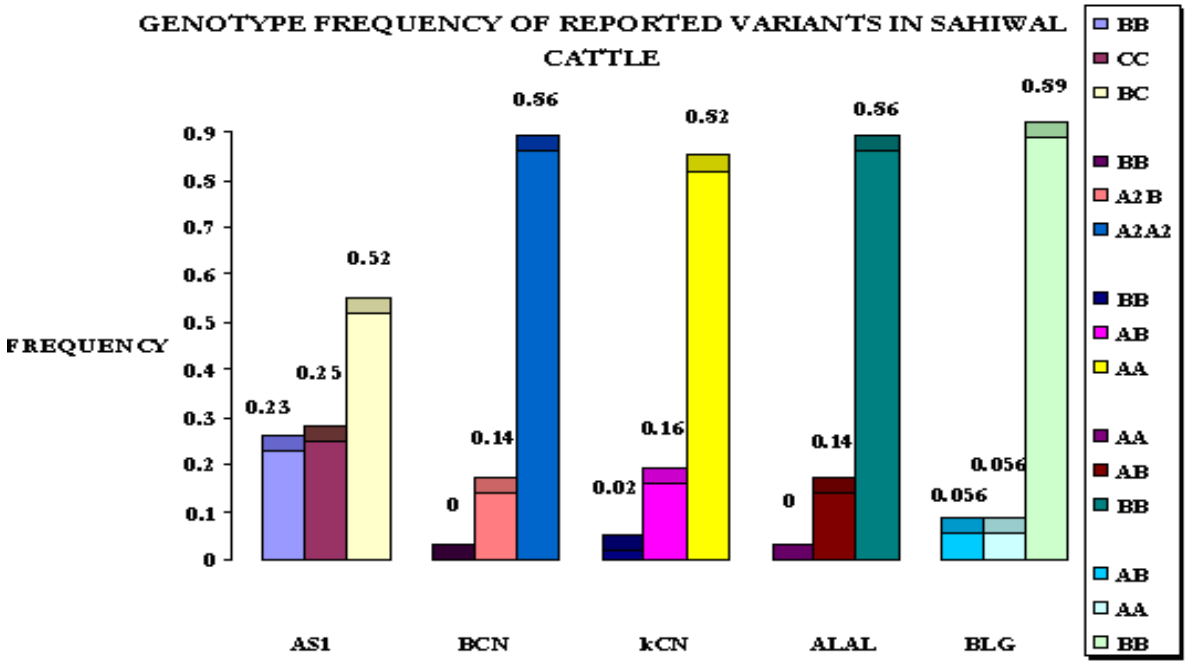

Figure 2. Genotype frequencies of reported variants. AS1: *alpha s1 casein*, BCN: *beta casein*, kCN: *kappa casein*, ALAL: *alpha lactalbumin* and BLG: *beta lactoglobulin*.

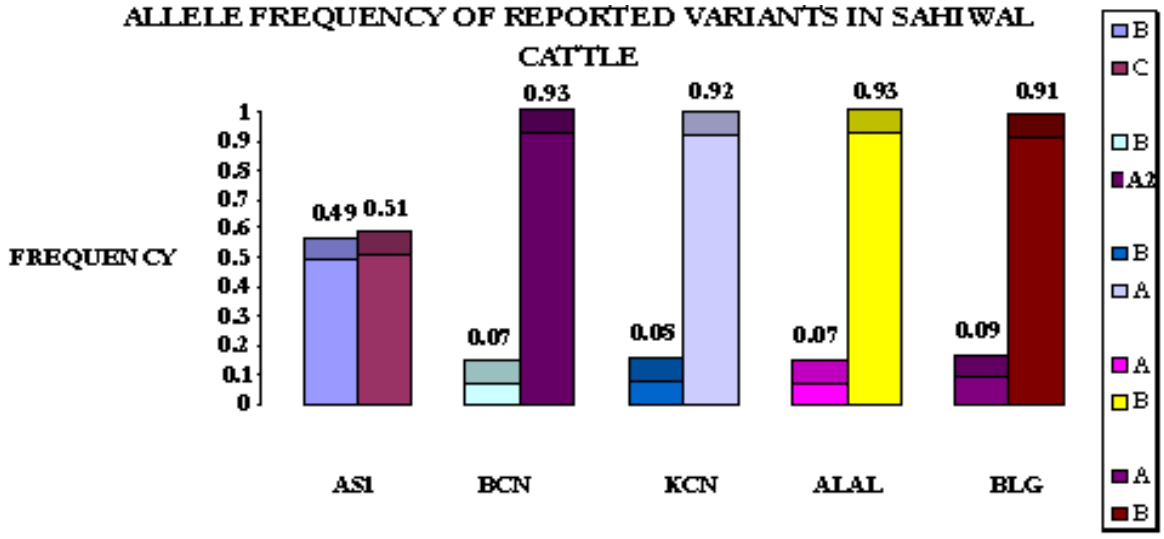

Figure 3. Allele frequencies of reported variants. AS1, *Alpha s1 casein*; BCN, *beta casein*; KCN, *kappa casein*; ALAL, *alpha lactalbumin*; BLG, *beta lactoglobulin*.

s1 casein, *beta casein*, *kappa casein*, *alpha lactalbumin* and *beta lactoglobulin* in Sahiwal cattle breed, respectively.

In *alpha s1 casein*, the genotype frequencies of Sahiwal animals were 0.23, 0.25 and 0.52 for BB, CC and BC genotypes, respectively. Alpha s1casein locus showed an approximately even distribution of alpha s1casein B (0.49) and C (0.51) alleles in Sahiwal cattle. Most common allelic forms observed in different breeds are B and C. The present data agree with previous reports (Aschaffenburg, 1968; Aschaffenburg et al., 1968) that predominance of the *alpha s1 casein* C allele in the Sahiwal cattle (*B. indicus*) contrasts with the high frequency of the *alpha s1 casein* B allele (90 to 95%) in *Bos taurus* breeds. A frequency close to 0.9 was

reported for the C variant in *B. indicus* (Ivana and Marco, 1997) and *Bos grunniens* (Eigel et al., 1984) while in *B. taurus* its frequency ranges from 0.2 to 0.4 (Baker and Manwell, 1980; Baranyi, 1992; Golijow et al., 1999; Yasemin and Cengiz, 2006). This asymmetric distribution in *B. indicus* breeds and European cattle breed has been explained by the different processes of domestication to which these animals were submitted (Grosclaude et al., 1974). Ivana and Marco (1997) studied frequency distribution at *alpha s1 casein* locus in Brazilian Zebu cattle (Gyr, Guzerat, Sindi, Nelore) and reported predominance of allele B (0.000 - 0.136) over C allele (0.864 - 1.000). Caroli et al. (2008) also reported dominance of B allele (0.8) over C allele (0.08) in Carora cattle. Creangă et al. (2010) reported allele B is more frequently encountered with a higher frequency than 0.7 and allele C had the frequency of 0.2 for the animals of Romanian Grey Steppe Breed.

At β-CN locus, A_2A_2 and A_2B genotypes had frequencies of 0.14 and 0.86, respectively (Figure 2). B allele was less frequent (0.07) and the A_2 allele more frequent (0.93) within the *beta casein* locus (Figure 3). There was no A1 genotype in animals under study. The present data agree with previous reports (Ng-Kwai-Hang et al., 1984; Ivana and Marco, 1997; Malik et al., 2000; Jann et al., 2002) that indicate predominance of the β-CN A_2 allele in the Sahiwal cattle (*B. indicus*). Mishra et al. (2009) also reported absence of A1 allele in most of Indian cattle breeds which is predominant in most of *B. taurus* breeds (Kaminski et al., 2007). The frequency of A1 allele in different breeds varies between 0.01-0.06 (Guernsey), 0.09-0.22 (Jersey), 0.31-0.66 (Holstein), 0.43-0.72 (Ayrshire) and 0.71 (Danish Red) as reviewed by Kaminski et al. (2007). Frequency of B allele in Sahiwal cattle (0.07) is close to Polish Red and Red Danish dairy cattle (0.06) as reported by Erhardt et al. (1998).

Recently Ganguly et al. (2013) reported frequency distribution at *beta casein* locus in Frieswal (HF X Sahiwal Crossbred) hence allele A1 is hypothesized to be of *B. taurus* origin rather than *B. indicus.* The frequency of A2 and A1 allele was 0.65 and 0.35, respectively and this high frequency of A1 in frieswal is due to crosses between Holstein (*B. taurus*) and Sahiwal (*B. indicus*). Olenski et al. (2010) have reported the frequencies of A1 (0.35) and A2 (0.65) alleles from Polish Holstein-Friesian bulls which are found to be very similar to Frieswal population.

Kappa casein (CSN3) is the most extensively studied milk protein in cattle which showed two predominant DNA genotypes AB and AA in present study with 0.16 and 0.82 frequencies respectively (Figure 2), two out of 120 animals studied were with CC genotype (frequency = 0.02). Allele A was more frequent in Sahiwal cattle than allele B, that is, 0.92 and 0.08, respectively (Figure 3) corroborating several findings (Ng-Kwai-Hang et al., 1984; Pinder et al., 1994; Kemenes et al., 1999, Golijow et al., 1999; Lara et al., 2002; Yasemin and Cengiz, 2006; Alipanah et al., 2008) that observed this in *B. indicus* and *B. taurus* . The genotyping results are also similar to earlier studies reported for Korean native cattle, Japanese brown, Angus, Hereford, Charolais and Holstein cows by Hung et al. (1995, 1998). Malik et al. (2000) reported similar observations in fourth-generation crossbred cattle (50% Holstein, 25% Jersey, 25% Zebu, that is, Haryana and Sahiwal cattle). Rachagani and Gupta (2008) studied the dominance of A allele in their study performed on Sahiwal and Tharparker breeds of Iindia. High frequency of A allele was also observed in Brazilin zebu Gyr, Guzerat and Nelore cattle (Azevedo et al., 2008). Bonvillani et al. (2010) and Ren et al. (2011) observed a higher frequency of allele A and lower frequency of allele B in Holstein cows. Contrary to these findings, Ceriotti et al. (2004) reported that allele B has higher frequency than allele A in *B. taurus* breeds as compared to *B. indicus* breeds. Moin and Supriyantono (2012) also compared allele frequencies at *kappa casein* locus in the breeds of *B. taurus* and reported higher frequency of B alleles than the breeds of *B. indicus*. Lukac et al. (2013) studied genotype distribution of *kappa casein* in Serbian Holstein-Friesian and found that this locus is at Hardy-Weinberg equilibrium ($P>0.05$), similar to that found by Hanusová et al. (2010) in Holstein cattle of Slovakia.

Alpha lactalbumin gene had AB and BB genotypes with frequencies of 0.14 and 0.86 while genotype AA was absent in this study (Figure 2). Alleles A and B of *alpha lactalbumin* were observed in this study with frequency of 0.07 for allele A and 0.93 for allele B (Figure 3). The observed higher frequency of the *alpha lactalbumin B* allele was consistent with results reported previously that *B. indicus* had high frequency for B allele than A allele compared to *B. taurus* breeds (Blumberg and Tombs, 1958, Bhattacharya et al., 1963; Bleck et al., 1993b; Ivana and Marco, 1997). Several workers have reported two genetic variants A and B at this locus in *B. indicus* and the droughtmaser (*B. indicus* x *B. taurus*) (Blumberg and Tombs, 1958; Bhattacharya et al., 1963; Bell et al., 1970; Osterhoff and Pretorious, 1966; Chianese et al., 1988), whereas a third variant, namely, C was reported in Bali cattle (*B. javanicus*) by Bell et al. (1981).

AB, AA and BB genotypes of *beta lactoglobulin* occurred at frequencies 0.056 for AB and AA each while BB genotype had highest frequency (0.89) in this study (Figure 2). The frequency of allele A and allele B in Sahiwal cattle were 0.09 and 0.91, respectively (Figure 3). These findings are consistent with studies of other authors (Aschaffenburg, 1964; Ivana and Marco, 1997; Litwinczuk and Krol, 2002; Celik, 2003; Yasmin and Cengiz, 2006; Daniela et al., 2008) who observed that allele A has less frequency than B allele in *B. taurus* as well as in *B. indicus* breeds. Karimi et al. (2009) showed that allele frequency of B allele (0.9125) was higher than that of the A allele 0.0875). In contrast to these findings Heidari et al. (2009) reported allele (frequencies of A and

Table 2. 1st lactation record and observed milk protein genotypes.

Protein	Genotype	Mean	Standard deviation	Significance
α-S1 Casein	BB	1952.8	610.93244	0.555 NS
	BC	1715.0	724.01986	
	CC	1636.8	671.68244	
β-Casein	A2A2	1727.7	602.71792	0.188 NS
	A2B	2027.7	802.18974	
κ-Casein	AA	1935.7	465.12855	0.041 S
	AB	2357.5	619.92349	
α-Lactalbumin	BB	1638.2	287.13450	0.068 NS
	AB	1879.4	396.66632	
β-Lactoglobulin	BB	1728.7	683.86772	0.872 NS
	AB	1936.4	166.05696	
	AA	1906.7	1402.89278	

NS: Non-Significant, S: Significant.

Table 3. 2nd lactation record and observed milk protein genotypes.

Protein	Genotype	Mean	Standard Deviation	Significance
α-S1 Casein	BB	2780.6	825.22309	0.823 NS
	BC	2746.6	937.67345	
	CC	2975.0	1049.21113	
β-Casein	A2A2	2529.6	964.81080	0.671 NS
	A2B	2379.2	635.35901	
κ-Casein	AA	2523.0	811.96566	0.047 S
	AB	3111.8	950.83972	
α-Lactalbumin	BB	2542.1	1000.78859	0.073 NS
	AB	3313.4	2015.84518	
β-Lactoglobulin	BB	2623.6	891.98183	0.910 NS
	AB	2343.0	1319.46125	
	AA	2543.5	1191.47493	

NS, Non-significant; S, significant.

B in Holstein cattle as 0.53 and 0.47, respectively. Lucak et al. (2013) reported similar findings for the Serbian Holstein Friesian cattle that *beta lactoglobulin* locus fitted with Hardy-Weinberg equilibrium (P<0.05), and was similar to that demonstrated by Gouda et al. (2011) in Egyptian Holstein cattle, and Ren et al. (2011) in Chinese Holstein and Jersey cows.

Genotype effects

Data published in research reports regarding correlations between the *alpha s1 casein* gene polymorphism and the milk traits is controversial that may partly be due to the differences in parameters used and/or variety in cattle breeds. In our study on Sahiwal cattle at *alpha s1 casein* locus all three variants studied (BB, BC and CC) had no effect ($p > 0.05$) on milk 1st lactation, 2nd lactation, (Tables 2 and 3). According to other publications (Ng-Kwai-Hang et al., 1984; Aleandri et al., 1990) *alpha s1 casein* BB genotype correlated with higher milk production than those with either AB or BC genotype. Results of Hristov et al. (2013) also agree with the dominance of the B allele over the C allele relative to the milk production. On the other hand Havliček (1996) and Micinski et al. (2007)

reported superiority of the heterozygous BC genotype with reference to milk yield.

In this study, *beta casein* reported variant had no effect ($p > 0.05$) on 1st lactation, 2nd lactation milk yield (Tables 2 and 3). There are similar findings (Aleandri et al., 1990; Mao et al., 1992; Famula and Medrano, 1994) which agree with our data. But according to Ng-Kwai-Hang et al. (1984), Bovenhuis et al. (1992), Ortner et al. (1995) and Cardak (2005), the *beta casein* genotypes had an effect on milk. Morris et al. (2005) showed superiority of A2 genotype with reference to milk yield in Holstein cattle. Tolenkhomba and Yadav (2012) also studied *beta casein* genotypes in Indian Sahiwal cattle and showed that cows with AB genotype produced more milk (11.81±2.10) than those with AA genotype whose values were 6.48±0.41. However genetic characterization of *beta casein* is important because A2 allele that is predominant in Sahiwal cattle is beneficial with reference to human health (Chatchatte et al., 2001).

Kappa casein has significant effect ($p < 0.05$) on 1st and 2nd lactation in this study (Tables 2 and 3) which agrees with studies of other authors (Ng-Kwai-Hang et al., 1986; Mao et al., 1992; Cardak, 2005) who suggested that the *kappa casein* is associated with high production for milk during the first lactation, but there is difference in genotypes effecting milk production and even some studies indicated no effect at all (Ng-Kwai-Hang et al., 1990; Lundén et al., 1997). In our study, the animals with genotype AB had a higher 1st and 2nd lactation milk yield than those with genotypes AA and BB. A similar effect had been noticed by Ng-Kwai-Hang et al. (1986) Bovenhuis et al. (1992) and Hirstov et al. (2013) who showed that animals with *kappa casein A*B genotype were better milk producers than either of the animals homozygous for this gene in Ayrshire, Holstein, Jersey, brown Swiss, Canadienne and Guernsey and Bulgarian Rhodopean cattle breeds. Contrary to these findings, Gonyon et al. (1987), Curi et al. (2005), Cardak (2005), Sitkowska et al. (2008) and Ahmadi et al. (2008) reported AA genotype was associated with higher milk production than BB. In contrast, Lin et al. (1986), Van Eenennaam and Medrano (1991), Mao et al. (1992) and Rachagani (2008) reported BB genotype to be significantly affecting milk production than those with genotypes AA and AB. However, Ikonen et al. (1999) showed that the *kappa casein* genotypes had no distinct effect on milk production, which was in agreement with previous studies (Ng-Kwai-Hang et al., 1986; Gonyon et al., 1987; Aleandri et al., 1990; Bovenhuis et al., 1992; Bovenhuis et al., 1994; Famula and Medrano, 1994). This is indicative of variations among species, environments and management practices adopted at different farms.

In Sahiwal cattle, *alpha lactalbumin* did not have significant effect in 1st lactation ($p = 0.068$) and 2nd lactation ($p = 0.073$) on milk production, however, the p values were very close to significance level (Tables 2 and 3). Our results revealed that animals having AB genotypes had a higher average milk yield than the animals with BB genotypes (Tables 2 and 3). Hence our results are consistent with findings of Bleck and Bermel (1993b) who reported that *alpha lactalbumin* (+15) A variant was associated with greater milk yield. Sashikanth and Yadev (2011) also reported that BB genotype in zebu cattles (Sahiwal, Hariana, Tharparkar) showed higher milk yield. Zhang et al. (2007) screened Chinese Holstein cattle for *alpha lactalbumin* locus and found no significant association between the two genotypes found and milk production traits in these cattle. Recently Zhou et al. (2013) identified single nucleotide substitution C→T (α-LA2516) at position 2516 of the α-LA gene in Chinese Holstein cattle with frequencies of T and C as 0.67 and 0.32, respectively. There was no significant association between genotypes resulting from this SNP and production traits in cattle. In a future study, increase in number of samples may give significant findings in Sahiwal cattle with reference to *alpha lactalbumin*.

There was no significant effect ($p > 0.05$) of *beta lactoglobulin* genotype on the 1st lactation, 2nd lactation in this study (Tables 2 and 3). Our data agree with the results of Lunden et al. (1997) and Ojala et al. (1997) who reported no significant associations of different *beta lactoglobulin* genotypes on milk production. Nevertheless, there are also reports for the positive influence on the milk quantity of all the genotypes, for example, Pupkova, (1980) and Cardak, (2005) reported that cows having AB genotype produce more milk than cows of AA and BB genotypes, however, Aleandri et al. (1990), Bovenhuis et al. (1992) and Ikonen et al. (1999) observed the rare *beta lactoglobulin* genotype AA was associated with the highest milk production. Similar results describing effects of the *beta lactoglobulin* genotypes on milk production traits that were observed (Ikonen et al., 1999) and have been frequently reported (Ng-Kwai-Hang et al., 1984; 1992; Mao et al., 1992).

Heidri et al. (2009) reported that cows with the AA genotype produced more milk than animals with the BB genotype ($P< 0.006$). Contrary to these findings, Jairam and Nair (1983) and Hirstov et al. (2013) showed that the BB genotype determines higher milk production. Ahmadi et al. (2008) reported strong association between BB genotype and protein percentage while there was no association between *beta lactoglobulin* genotypes and milk yield or milk fat percent. Chi square tests showed significant association between genotypes and milk yield in this study (Table 4).

Genotyping technique

The most commonly used techniques used for DNA typing milk protein genes are restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP). These techniques either require high quality and quantity of DNA or the low detection sensitivity when amplicon sizes exceed 200 bp. To milk

Table 4. Chi square test for association of genotypes with milk yield.

Protein	Value	df	p-Value
α-S1 Casein	1.947E5	119	0.000
β-Casein	1.525E5	119	0.000
κ-Casein	1.412E5	119	0.000
α-Lactalbumin	1.597E5	119	0.000
β-Lactoglobulin	2.093E5	119	0.000

df, Degree of freedom; p-value, 0.05.

overcome these limitations SNaPshot genotyping was optimized to type genetic variants in all five milk proteins under study. In a future study, all five variants of major proteins will be multiplexed to make this technique cost effective and hence more efficient for genotyping in Sahiwal cattle.

Conclusion

In the present study, we designed a strategy to avoid long and costly traditional selection methods for dairy purposes in Sahiwal cattle. We worked on reported variants of *alpha s1 casein*, *beta casein*, *kappa casein*, *alpha lactalbumin* and *beta lactoglobulin* in Sahiwal cattle. Statistically significant differences were observed in *kappa casein* genotypes AA (AA) and AB (AC), that is, genotype AB had more milk yield than genotype AA in 1st lactation (422 kg) and 2nd lactation (612 kg), respectively. Results of our studies observe that A allele of *kappa casein* is near fixation limit in Sahiwal cattle so it would be difficult to increase frequency of B allele but we assume that selection of bulls with AB and BB genotype for *kappa casein* may help to increase the frequency of B allele in the Sahiwal cattle population of Pakistan. The favorable allele B of *alpha lactalbumin* in Sahiwal cattle breed is already found to be near fixation limit, hence we do not need to alter frequency. However, large population size of Sahiwal cattle must be studied to observe the statistically significant effects of AB genotypes.

Indirect milk tests are limited to mature lactating females and indirect genotyping of sires. These tests require extremely long times to obtain results which makes them impractical for establishing breeding development programs aimed at increasing the frequency of desired milk protein alleles in progeny. Breeding cows are selected on few performances and even before they start their first lactation, which is costly and does not always, result in precise identification of the animal breeds that produce higher milk yield. In dairy cattle, selection of bulls is mostly based on progeny-tests which involve recording the performances of large groups of female off springs of these bulls. A SNP genotyping method that was optimized in this study is simple and efficient which can be used not only for the selection of sires for artificial insemination but also for selection of immature cows and for pre-implantation embryos for embryo transfer. To the best of our knowledge, this is the first comprehensive study involving milk proteins in the *B. indicus* Sahiwal cattle breed of Pakistan.

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