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Analysis of allelic pattern across milk trait genes in native cattle adapted to high altitude region of Leh-Ladakh

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ABSTRACT

Ladakhi, the native cattle from Ladakh region of India have developed over the years under natural selection and can survive well at extreme climatic conditions, viz. high altitude, huge barren lands, low temperature (≤–20°C) and hypoxic conditions. Even at extreme survival conditions, this cattle provides around 2–5 kg of milk. This highly evolved germplasm might possess unique alleles or combinations of alleles, hence attempt was made to study the frequency of allelic variants at important candidate gene loci affecting dairy traits. The observed distribution pattern of allele frequencies were different from taurine but in accordance with other Indian native cattle breeds indicating maintenance of indicine characteristics and near absence of taurine influence/introgression effect on this naturally evolved germplasm. Further, variant E at κ-*CN* locus and two novel variants at *BTN-3* loci were also observed. The present findings helped to understand the unique Ladakhi cattle population with respect to polymorphism and distribution of various alleles in candidate genes related to milk traits.

Keywords: Candidate gene, Genetic polymorphism, High altitude, Ladakhi cattle, Leh-Ladakh, Milk trait

Characterization of candidate genes influencing traits of economic importance are of paramount importance for understanding the molecular basis of performance traits in different livestock breeds. To date, polymorphism in milk protein genes and their association with milk quality and quantity has been reported in different Indian native as well as exotic cattle breeds (Grosclaude 1988, Jeichitra *et al.* 2003, Caroli *et al.* 2004, Kucerova *et al.* 2006, Sodhi *et al.* 2007, Mir *et al.* 2014), but their status (gene frequencies and gene diversity) is still unknown in many of the naturally evolved lesser known cattle population of India. One such cattle population is Ladakhi cattle, from Ladakh region, situated roughly between 32 to 36 degree north latitude and 75 to 80 degree east longitude and altitude ranging from 2,300 to 5,000 meters above sea level. Ladakhi cattle can well survive at extreme conditions, viz. high altitude (5,753 m), huge barren lands without vegetation and assured water supply, low temperature (below –20°C in winter season) and hypoxic conditions. Inspite of extreme climatic conditions, subsistence on poor quality feed and low availability of water, this local cattle provides around 2–5 kg of milk per day and thus plays an important role in the life of local people (Mukesh *et al*. 2017). Animals of Ladakhi cattle are well built, short statured (75–80 cm height) with cylindrical body, small hump, and long hairs with glossy sheen. Body colour varies from black, brown to black with white patches (Fig. 1). Although, cattle in the Ladakh region is the lifeline of local people as land resources are meagre, there has been no systematic study to characterize this local germplasm. Further, as Ladakhi cattle has developed over the years under natural selection and is highly evolved germplasm, it is hypothesized that it might possess unique alleles or combinations of alleles that are different from other indigenous and taurine cattle breeds. To understand trait specific gene characterization, allelic profiling at important candidate gene loci is an important strategy. In this line of studies, for instance, B variants of beta-casein (Heck *et al.* 2009), kappa-casein (Caroli *et al.* 2004, Hallen *et al.* 2008, Heck *et al.* 2009) and β*LG* (Hallen *et al.* 2008; Daniela *et al.* 2008) have been observed to be associated with an increase in milk casein content and cheese yield/quality in various cattle breeds, whereas E variant of kappa-casein (Hallen *et al.* 2008) was associated with a decrease in casein content.. The present study was therefore, undertaken to delineate the genetic polymorphism in candidate loci for milk production and composition including Kappa-casein (κ-*CN*), Beta-casein (β-*CN*), Beta-lactoglobulin, Alpha-lactalbumin, bovine Growth Hormone (*bGH*), Pituitary transcription factor (*Pit*-1), Prolactin (*PRL*), Butyrophilin1,3 (*BTN*-1,-3) and Diglycerol Acyltransferase (*DGAT-1*) affecting milk production and composition for functional traits across Ladakhi cattle using

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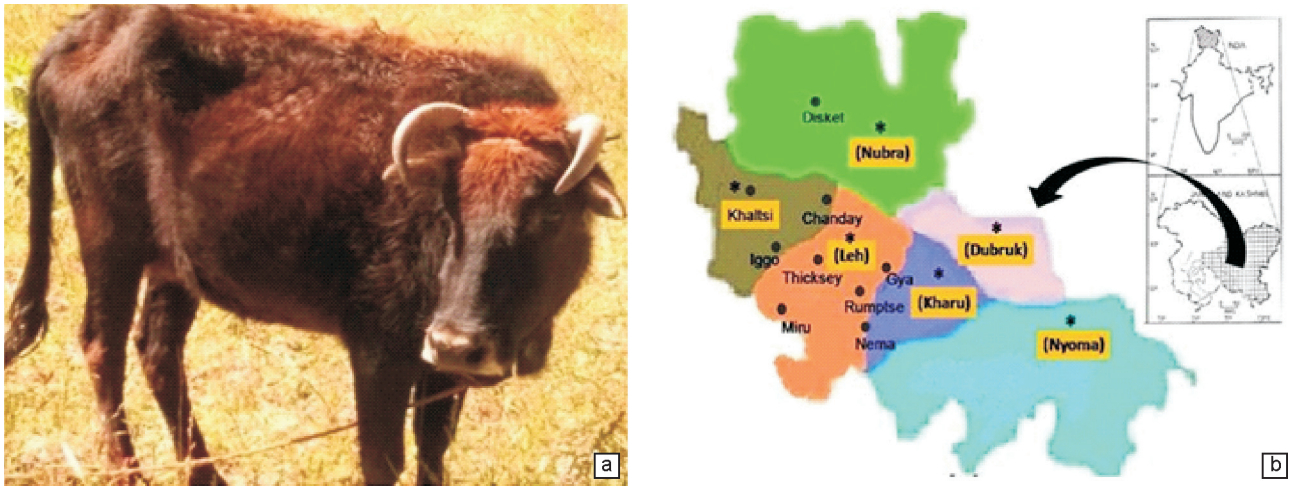

Fig. 1. Distribution and sampling sites of Ladakhi cattle (a) Typical Ladakhi cow; (b) Sampling sites in breeding tract. (Star in the map depicts blocks while dark circles depicts the sampling sites).

PCR-RFLP (PCR-Restriction fragment length polymorphism) technique.

MATERIALS AND METHODS

Sampling and DNA extraction: Blood samples (8–9 ml) were collected from 72 unrelated and true to breed animals from 11 different villages of the breeding tract (Leh-Ladakh region) using vacutainer tubes containing EDTA as anticoagulant. The sampling sites are depicted in Fig.1. The samples were brought to lab at 4°C. Genomic DNA was extracted using HiPur^{ATM} SPP Blood DNA kit (HIMEDIA, India). The quality and concentration of extracted DNA samples was assessed by electrophoresis on 0.6% agarose gel in 1× TAE buffer and the O.D. 260/280 was estimated using Nanodrop 1000 (Eppendorf, Germany). The samples having intact band on agarose gel and with O.D. 260/280 between 1.8–2.0 were used for further analysis.

Molecular genotyping: To delineate the allelic variants across the selected candidate genes, the DNA samples were genotyped by PCR-RFLP approach. The primers for amplification of genomic region of correspondent gene covering the specific polymorphic region were synthesized based on available literature (Table 1). Amplification reactions were performed in a final volume of 25 µl containing 80–100 ng genomic DNA, 5 pmol of each primer pair, 1× reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each

Table 1. Primer sequences for amplification of selected region of candidate genes

Candidate gene	Primer sequence	Genomic region	Reference
k-CN	F 5′-AGCGCTGTGAGAAAGATG-3′ R 5′-GTGCAACAACACTGGTAT-3′	Exon 4	(Sodhi *et al.* 2010)
β-CN/TaqI	F-5′-CCAGACACAGTCTCTAGTCTATCCC -3′ R-5′- CAACATCAGTGAGAGTCAGGCTCCG -3′	Exon 7	(Sodhi *et al.* 2012)
β-CN/MspI	F-5′-GAGTCGACTGCAGATTTTCAACATC AGTGAGAGTCAGGCCCTG 3′ R-5′ CCTGCAGAATTCTAGTCTATCCCTTCCC TGGGCCCATCG 3′	Exon 7	(Sodhi *et al.* 2012)
β-LG	F-5′ TGTGCTGGACACCGACTACAAAAAG- 3′ R-5′ GCTCCCGGTATATGACCACCCTCT- 3′	Exon 4-Intron 4	(Jairam *et al.* 1983)
α-LA	F-5′ TTGGTTTTACTGGCCTCTCTTGTCATC-3′ R-5′ TGAATTATGGGACAAAGCAAAATAGCAG-3′	5’ region–Intron1	Schlee and Rottamann, 1992.
bGH	F-5′- CCACGGGCAAGAATGAGGC-3′ R-5′- TGAGGAACTGCAGGGGCCCA-3′	Intron 3	(Sodhi *et al.* 2007)
Pit-1	F-5′- CAATGAGAAAGTTGGTGC - 3′ R-5′-TCTGCATTCGAGATGCTC - 3′	Exon 5 and 6	(Mukesh *et al.* 2008)
PRL	F-5′- CGAGTCCTTATGAGCTTGATTCTT-3′ R-5′- GCCTTCCAGAAGTCGTTTGTTTTC-3′	Exon 3	(Sodhi *et al.* 2011)
*BTN*1	F-5′- TGGAGCTCTATGGAAATGGG -3′ R-5′- TACCCAACAGGAAGAAACAG-3′	Exon 7	(Lee *et al.* 2002)
*BTN*3	F-5′–CTGAAGTTCCCGACAAACTCG-3′ R-5′- CTCTGCATCTTCACCCACCAC-3′	Exon 2–Exon 3	(Lee *et al.* 2002)
DGAT1	F-5′- GCACCATCCTCTTCCTCAAG-3′ R-5′- GGAAGCGCTTTCGGATG-3′	Exon 7	(Tantia *et al.* 2006)

dNTPs mix, and 1.0 U of *Taq* polymerase (Invitrogen, USA). The thermal cycling conditions used for amplification of desired region of the respective genes have been listed in Table 2. Amplification was verified by electrophoresis of an aliquot of PCR product on 2% agarose gel using a 100 bp ladder as a molecular weight marker to confirm of the size of the PCR products. For PCR-RFLP analysis 10 µl of each PCR product (~1 µg) was digested with 1 unit of specific restriction endonuclease by incubating at 37°C for 3–4 h followed by 20 min of inactivation at 65°C. The details of the restriction enzymes used are provided in Table 2. The resultant fragments were separated by electrophoresis on agarose gel stained with ethidium bromide in Ix TAE buffer for 30 minutes and visualized under UV light.

Statistical analysis of PCR-RFLP data: Allele/genotype frequencies for each gene were determined to understand the distribution pattern of genetic variants in Ladakhi cattle populations. Genotypes were determined by direct counting while the frequency of the specific allele was calculated as the sum of the frequency of the homozygous plus half the frequency of heterozygous genotypes as a proportion of the total animals.

Table 2. PCR annealing temperature, expected fragment size, and restriction enzymes used for different candidate genes

Candidate gene	PCR conditions			Restriction Enzyme	Length
	Temperature		Time		
	95°C		45 sec		
κ-CN	58°C	30 cycles	60 sec	*Hind*III&	935 bp
	72°C		60 sec	*Hae*III	
	94°C		30 sec		
	63°C	30 cycles	30 sec	*Taq*I	251 bp
β-CN	72°C		20 sec		
	94°C		30 sec		
	60°C	35 cycles	30 sec	*Msp*I	208 bp
	72°C		30 sec		
β-LG	94°C		75 sec		
	60°C	35 cycles	60 sec	*Hae*III	166 bp
	72°C		75 sec		
βα-LA	92°C		75 sec		
	60°C	30 cycles	60 sec	*Msp*I	306 bp
	72°C		75 sec		
	94°C		60 sec		
Pit-1	55°C	30 cycles	60 sec	*Hinf*I	660 bp
	72°C		60 sec		
	94°C		30 sec		
PRL	59°C	30 cycles	40 sec	*Rsa*I	156 bp
	72°C		20 sec		
	94°C		40 sec		
bGH	60°C	30 cycles	40 sec	*Msp1*	329 bp
	72°C		30 sec		
	94°C		60 sec		
	60°C	30 cycles	40 sec	*HaeIII*	576 bp
BTN	72°C		60 sec		
	94°C		60 sec		
	61°C	30 cycles	50 sec	*Taq*I	576 bp
	72°C		60 sec		
	94°C		40 sec		
*DGAT*1	55°C	35 cycles	40 sec	*Cfr*I	411 bp
	72°C		30 sec		

RESULTS AND DISCUSSION

Polymorphism at kappa-casein (*k-CN*) *gene:* The PCR amplified product of 935 bp length corresponding to the region harboring most of the polymorphic loci was investigated to ascertain the allelic/genotypic distribution of *k-CN* variants. This region has been reported to carry polymorphic sites for *Hind*III and *Hae*III restriction enzymes. Digestion with *Hind*III and *Hae*III yielded different restriction pattern and five different genotypes (AA, AB, BB, BE and AE) were identified (Fig. 2). On analysis, it was observed that AB genotype (fragments of 935, 520 and 415 bps with *Hind*III and 641 and 295 bps with *Hae*III) was most commonly distributed (0.53) followed by AA genotype (935 bp with *Hind*III and 641 and 294 bps with *Hae*III) with a frequency of 0.28. The other genotypes BE, BB, AE were observed with a frequency of 0.10 or less. The representative profile of restriction fragments generated at *k-CN* locus is depicted in Table 3. The allelic pattern indicated the A allele (0.57) as most predominant, followed by B (0.36) and E (0.06) alleles. Amongst the eleven variants of *k-CN* identified in cattle (Farrell *et al.* 2000), A and B are the most common protein variants that differ at codons 136 (A: Thr, ACC and B: Ile, ATC) and 148 (A: Asp, GAT and B: Ala, GCT) of exon-IV (Ron *et al.* 1994). Studies have indicated the B variant to be significantly associated with content and yield of milk protein and fat, as well as having a role in curd and cheese making properties (Heck *et al.* 2009). The frequency of B allele which is considered as preferred allele was comparatively low (0.36) in Ladakhi cattle, however it is quite higher than the frequency value (0.092) observed for other Indian cattle breeds (Sodhi *et al.* 2010). Similar higher values for B allele has been observed in Simmental (0.35) and Jersey (0.65) (Regitano *et al.* 2000, Shetty *et al.* 2006)

Indian cattle breeds differed significantly from the taurine cattle breeds with respect to frequency distribution of *k-CN* genotypes, where, the preferred BB genotype was more prevalent. However, high frequency of A allele and AA genotype at *k-CN* was also observed in Brazilian zebu Gyr and Nellore cattle (Kemenes *et al.* 1999). Biase *et al.* (2005) found an allele frequency of above 0.91 for Allele A in Brazilian Nellore cattle. Similarly, Golijow *et al.* (1999) reported high frequency of A allele in Argentine Creole (AC) and Argentine commercial Holstein (AH) cattle). The major reason for high frequency of A alleles of *k-CN* in Brazilian zebu cattle was thought to be due to intensive selection for weight gain and meat production (Biase *et al*. 2005). Conversely, the Indian native cattle breeds like Ladakhi cattle are known to be evolved naturally with almost no artificial selection pressure for productive traits, showed comparatively lower frequency of A allele and AA genotype.

Further, allele E observed with a frequency of 0.06 in Ladakhi cattle has not been reported for other Indian cattle breeds. E variant of kappa-casein has been associated with

a decrease in milk casein content and cheese yield/quality in taurine cattle breeds (Hallén *et al.* 2008), however no such report is available for *B. indicus* breeds. The presence of E allele in few animals might be attributed to admixture of Ladakhi cattle with Jersey or Holstein Frisian cattle especially in the surrounding areas of Leh city.

Polymorphism at beta-casein (β-*CN*) *gene*: In *â-CN* gene, two SNPs, viz. C8101A and C8267G in VIIth exon were targeted. The polymorphism C8101A leads to amino acid change from proline to histidine at position 67 and variants are known as A1 and A2. Restriction digestion of the amplified product in Ladakhi cattle resulted in two genotype, homozygous A2A2 and heterozygous A1A2 with a frequency of 0.83 and 0.17 respectively. Genotype A1A1 was not observed in any of the animal. The corresponding allelic frequency was 0.92 and 0.08 for A2 and A1 allele respectively, thus indicating the predominance of A2 allele. The frequency spectra is similar to all other Indian native cattle breeds (Sodhi *et al.* 2012). Conversely, the frequency of A1 allele has been observed to be higher in taurine breeds (Mishra *et al.* 2009). The A1/A2 allele of β-*CN* holds special importance as due to the presence of histidine at amino acid 67 position, gastrointestinal proteolytic digestion of A1 β-*CN* (raw/processed milk) releases a 7 amino acid bioactive peptide 'opioid' called beta-casomorphin 7 (BCM-7) in small intestine, while proline in A2 milk at 67 position prevents the split at this particular site and generates nine amino acid peptide BCM-9 (Kostyra *et al.* 2004). BCM-7 is suggested to be associated as a risk factor for human health hazards as it can potentially affect numerous opioid receptors in the nervous, endocrine and immune system. It is also known to be an oxidant of low dietary lipoproteins (LDL) and oxidation of LDL is believed to be important in formation of arterial plaque. A number of recent studies link A1 beta-casein/BCM-7 to adverse physiology and immunity (Kost *et al*. 2009, Ho *et al*. 2014, Jianquin *et al*. 2016)

Another nucleotide polymorphism (C/G) studied in β-*CN* gene is at position 8267 (VII exon). *Msp*I restriction analysis of the PCR product yielded banding pattern corresponding to three different genotypes, viz. AA genotype with two fragments (208 and 25 bps), AB genotypes with three fragments (233, 208 and 25 bps) and BB genotype with one fragment (233 bp) (Fig.2). The genotype and allele frequencies of β-*CN* variants in Ladakhi cattle breeds are presented in Table 3. The analysis clearly indicated the abundance of A allele with a frequency of 0.97. Among the three genotypes, AA was most abundant with a frequency of 0.94 followed by AB with frequency of 0.06. None of the animal showed BB genotype.

Polymorphism at β-*lactoglobulin* (β-*LG*) *gene:* Beta-lactoglobulin (β-*LG*) is one of the major whey protein found

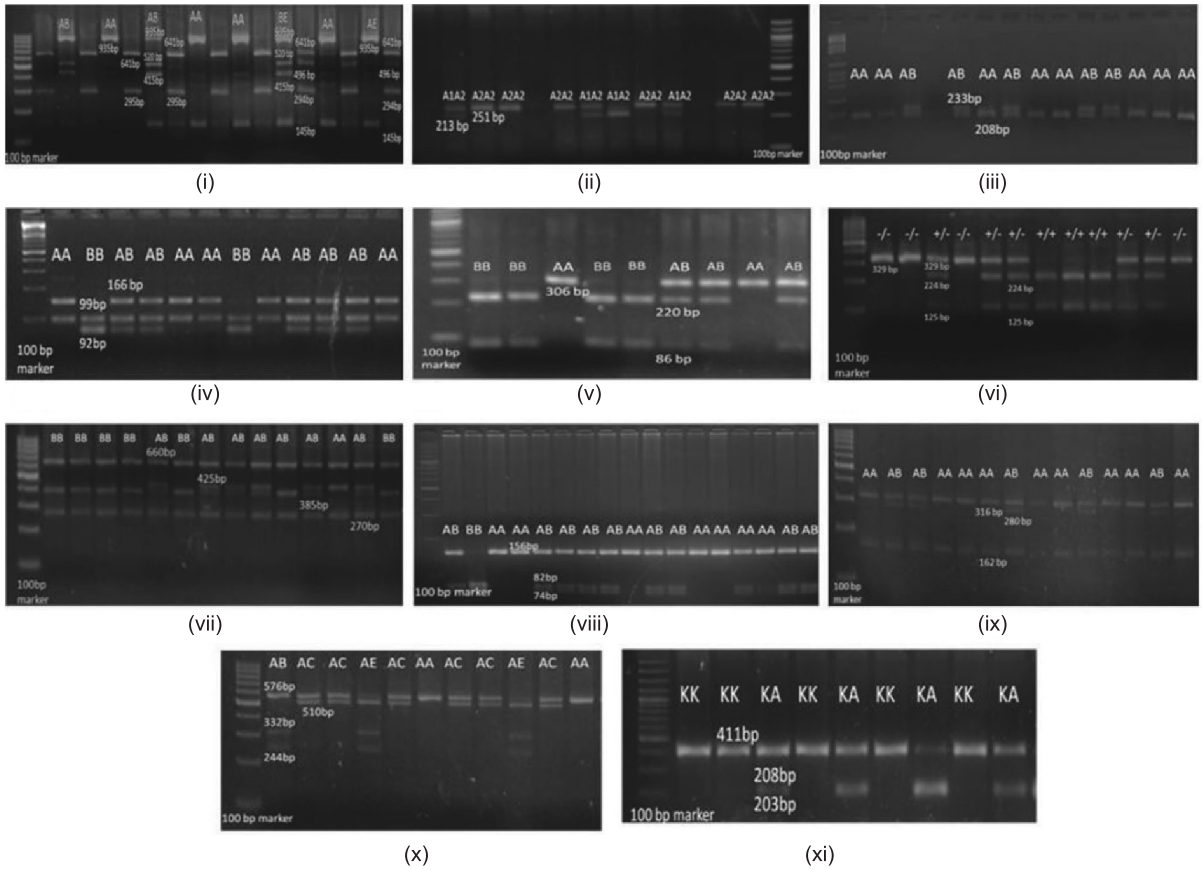

Fig. 2. Representative genotypes at analyzed candidate gene locus/loci. (i) κ-*CN/HindIII-HaeIII* loci (ii) β-*CN1/Taq/I* locus, (iii) β-*CN/MspI* locus (iv) β-*LG/HaeIII* locus, (v) *á–LA/MspI* locus (vi) *bGH/MspI* (vii) *Pit-1/HinfI* locus (viii) *PRL/RsaI* locus (ix) *BTN1/HaeIII* locus (x)*BTN3/TaqI* locus (xi) DGAT1/ *CfrI* locus.

Table 3. Genotypic and allelic frequencies of different candidate genes in Ladakhi cattle

Candidate gene	Genotype	Fragment sizes (bp)		Genotypic frequency	Allele	Allelic frequency
*β-CN/ Taq*1	A1A1A1A2	213, 38		0.00	A1A	0.08
	A2A2	251, 213		0.17	2	0.92
		251		0.83		
*β-CN/Msp*1	AAAB	208, 25		0.94	A	0.97
	BB	233, 208, 25		0.06	B	0.03
		233		0.00		
		HindIII	*HaeIII*			
	AA	935	641, 294	0.28	A	0.57
	AB	935, 520, 415	641, 294	0.53	B	0.36
k-CSN	BB	520, 415	641, 294	0.04	E	0.06
	BE	935, 520, 415	641, 496, 294, 145	0.10		
	AE	935	641, 496,294,145	0.04		0.50
	AA	148, 99		0.40	A	0.50
β-LG	AB	148, 99,74		0.20	B	
	BB	99,74		0.40		
	AA	306		0.01	A	0.18
α-LA	AB	306, 220, 70		0.33	B	0.82
	BB	220, 86		0.66		
	-/-	329		0.29	–	0.54
bGH	-/+	329, 224,125		0.51	+	0.46
	++	224,125		0.2		
	AA	660, 425, 270		0.03	A	0.20
PIT-1	AB	660, 425, 385, 270		0.34	B	0.80
	BB	660, 385, 270		0.63		
	AA	156		0.25	A	0.52
PRL	AB	156, 82, 74		0.54	B	0.48
	BB	82, 74		0.21		
	KK	411		0.59	K	0.79
*DGAT*1	KA	411, 208, 203		0.41	A	0.21
	AA	208, 203		0.00		
	AA	316		0.70	A	0.85
*BTN*1	AB	316, 280, 162		0.30	B	0.15
	BB	280, 162		0.00		
	AA	576		0.42	A	0.67
	AB	576, 332, 244		0.31	B	0.23
*BTN*3	BB	332, 244		0.08	C	0.08
	AC	576, 502		0.15	E	0.002
	AE	576, 502, 332, 244		0.04		

in the milk of ruminants species (Patel *et al.* 2008). PCR-RFLP analysis of β-*LG* region extending to 4th exon and intron was carried out to reveal the allelic pattern in the studied Indian cattle breeds. After digestion with *Hae*III restriction enzyme, the PCR product yielded banding patterns corresponding to three different genotypes, viz. AA genotype with two bands (166 and 99 bp), BB genotypes with three bands (99, 92 and 74 bp) and AB genotype with four bands (166, 99, 92 and 74 bp). The genotype and allele frequencies of *Hae*III-β-*LG* variants in Indian zebu breeds are depicted in Fig. 3. The data indicated predominance of both AB/, BB genotypes and A/B allele occurring with equal frequency of 0.40 and 0.50 respectively in the studied population. B allele of β-*LG* has been observed to be significantly associated with fat, protein, casein, total solid content and cheese yield (Hallén *et al.* 2008) whereas A allele is recognized for yield parameters (Strzalkowska *et*

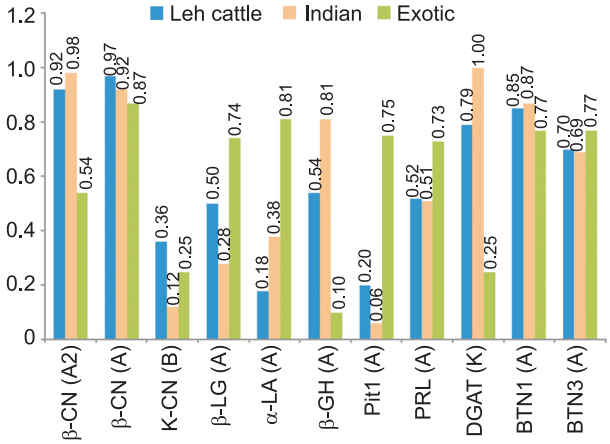

Fig. 3. Comparative allelic frequency distribution across different candidate genes loci in Ladakhi cattle *vis*-a-*vis* other Indian native (*B. indicus*) and taurine (*B. taurus*) cattle breeds.

al. 2002). In contrast to our data on Ladakhi cattle, high frequency of B allele (82.9%) and BB genotype (71.4%) was observed in Hariana cattle (Ganai and Bhat 2001) and Sahiwal (B=0.83; BB=0.61) (Rachagani *et al.* 2006). Some other reports also suggested the predominance of allele B in *B. taurus* as well as *B. indicus* breeds (Celik, 2003,Oner and Elmaci 2006, Daniela *et al.* 2008, Karimi *et al.* 2009, Mir *et al*. 2014). However, some of the cattle breeds especially Brazilian Simmental (Regitano *et al.* 2000), Guzerath, Nellore, Gyr (Kemenes *et al.* 1999)and Holstein cattle (Tsiaras *et al.* 2005) had almost equal frequency of A allele as that was observed for the Ladakhi cattle (0.50) in the present study.

Polymorphism at alpha-lactalbumin (α*-LA*) *gene:* Alpha-lactalbumin (α*-LA*), another whey protein involved in the synthesis of lactose in mammary gland. Genetic polymorphism of α*–LA* locus revealed typical restriction fragment pattern with two alleles (A, B) and three genotypes (AA, AB and BB) (Fig. 2). The genotype and allele frequencies of α*–LA* variants in Ladakhi cattle are listed in Table 3. The DNA restriction fragments obtained corresponding to AA, AB and BB genotypes were: 306 bp; 306, 220 and 70 bps; 220 and 86 bps, respectively. On analysis of the restriction pattern, genotypic frequency of 0.01, 0.33 and 0.66 was observed for AA, AB and BB genotype, respectively. Amongst allelic frequencies B allele was found to be the predominant allele with a frequency of 0.82. Similar trend of allelic distribution has also been observed in Hariana (Blumberg and Tombs 1958) and Sahiwal cattle (Osterhoff and Pretorius 1966) as compared to that of *B. taurus* breeds where allele A was predominant. Allele A of α*–LA* is widely considered as preferred allele as associated with higher milk, protein and fat yield in homozygous AA genotype (Bleck and Bermel 1993, Mao *et al.* 2004, Martin *et al.* 2002) considered as a valuable genetic marker for milk production traits in dairy cattle. Low frequency of AA genotype in Ladakhi and other Indian cattle breeds may require some strategic planning to increase the frequency of preferred allele in Indian cattle population. More importantly the high frequency of B allele in Indian native population could also be exploited for economic gains as this is known to be associated with higher protein and fat percentage (Bleck and Bermel 1993) and lower age at first calving (Jairam and Nair 1983).

Polymorphism at growth hormone (*bGH*) *gene:* The bovine growth hormone (*bGH*) plays an important role in growth, lactation and mammary gland development making it a promising candidate gene marker to study its effect on milk and growth related traits (Etherton and Bauman 1998). Allelic polymorphism at intron III region of *bGH* gene, involving a potential polymorphic *Msp*I site revealed 2 alleles (*Msp*I- and *Msp*I+) and 3 genotypes (-/-, +/- and +/+) across the investigated animals. The animals were designated as homozygous -/- when the amplified DNA fragment resulted in a single fragment of 329 bp (no digestion; TC) and scored as heterozygous +/- when characterized by the occurrence of three fragments, corresponding to 329, 224 and 105 bp (Fig. 2) and homozygous +/+ genotype resulted in two bands of 224 and 105 bp. The genetic profile at *bGH* locus in terms of *Msp*I genotype and allele frequencies is presented in Table 3. Allele A/*Msp*I (-) of *bGH* tend to increase milk, protein and fat yield, but decreases fat and protein percent (Zhou *et al.* 2005). For Ladakhi cattle frequency of *Msp*I (-) allele was higher (0.54) than that of *Msp*I (+; 0.46). The pattern of the *Msp*I allele frequency obtained in this study substantiates the few earlier studies reporting a higher frequency of the *Msp*I (-) allele in Sahiwal, 0.86 (Mitra *et al.* 1995); Brazilian Nellore, 0.82; and Ongole, 1.00 (Lagzi el *et al.* 2000) and 20 diverse Indian native cattle breeds (Sodhi *et al.* 2007). When compared with *Bos taurus* breeds, the *Msp*I (-) allele frequencies in Indian zebu cattle were quite high from those reported for taurine breeds from Northern Europe, Mediterranean countries, and America, where very low frequencies of the *Msp*I (-) allele and high frequencies of the *Msp*I (+) allele were detected. For example, reported frequency of *Msp*I (-) was 0.125 in the Holstein breed (Zhou *et al.* 2005); 0.00 in Hereford, 0.15 in Jersey, 0.14 in Angus (Lagzi el *et al.* 2000); 0.05 in Norwegian Red, and in Red Danish (Hoj *et al.* 1993). The evaluation of representative global data over the years demonstrated the average frequency of the *Msp*I (-) allele to be 0.10 overall, 0.39 for cattle breeds of Northern Euopean/USA, Mediterranean (Lagziel *et al.* 2000), and 0.86 in Indian cattle breeds (Sodhi *et al.* 2007). The high frequency of the *Msp*I (-) allele in Ladakhi and other Indian cattle and its decreasing values with increasing distance from the Indian subcontinent further reinforced the hypothesis that the *Msp*I (-) allele has an Indian derivation (Hoj *et al.* 1993).

Polymorphism at pituitary specific transcription factor (*Pit-1*) *gene:* The Pituitary specific transcription factor (*Pit-*1) is another important candidate gene that regulates the expression of growth hormone, prolactin and thyrotropin genes (Nelson *et al.* 1988). Mutation in *Pit-1* gene is associated with a marked decrease of both *GH* and *PRL* expression and proliferation of somatotropic and lactotropic cell lines (Castrillo *et al.* 1991). Allele A of *Pit-*1is associated with high milk and protein yield and is inferior for fat percentage in dairy cattle (Zwierzchowski *et al.* 2002). On screening *Pit-*1/*Hinf*I polymorphism in Ladakhi cattle samples, two alleles (A, B) and three genotypes (AA, AB and BB) were revealed (Fig. 2). The distribution pattern of A and B variants of *Pit-*1 in Ladakhi cattle showed higher frequency of B allele (0.8) and BB genotype (0.63) whereas frequency of allele A was found to be substantially low (0.2) (Table 3). Similar results were reported by Mukesh *et al.* (2008) in *Bos indicus* where the frequency of allele B was significantly high in all the Indian cattle breeds irrespective of its agroclimatic region. Similarly high frequency of B allele was also reported in Polish Black and White cattle (Dybus *et al.* 2004), Brazilian Gyr cattle (Mattos *et al.* 2003). Moody *et al.* (1995) also reported low frequency of allele A in Brahman cattle (0.10), Angus cattle

(0.45) and Holstein cattle (0.26).

Polymorphism at prolactin (*PRL*) *gene:* A silent A to G transition mutation at the codon for amino acid 103 in exon 3 of bovine *PRL* gene is responsible for polymorphic *Rsa*I site (Lewin *et al.* 1992). On restriction digestion analysis of 156 bp PCR product of exon 3 region of *PRL* gene with *Rsa*I enzyme, three genotypic patterns were observed, AA- 156 bp; BB- 82 and 74 bp and AB- 156, 82 and 74 bp (Fig.2). Genotype and allele frequencies of prolactin (*PRL-Rsa*I) gene variants revealed heterozygous AB genotype to be most prevalent (0.54) in Ladakhi cattle. Interestingly, the other two homozygous genotypes, AA and BB occurred at almost similar frequencies of 0.25 and 0.21, respectively (Table 3). The frequency distribution of A (0.52) and B (0.48) allele indicated their equal prevalence in the studied cattle population. The genotypic and allelic frequency spectra for Ladakhi cattle is similar to that observed for other Indian native cattle breeds (Fig.2). However, this profile was in sharp contrast to what had been reported for 96 Korean bulls (Jang *et al.* 2005). In their study, AA genotype was most predominant with a frequency of 0.781, followed by AB genotype (0.219). Also the allelic frequency reported by them differed significantly from the present findings. The frequencies for A and B allele was reported to be 0.891 and 0.109, respectively as against 0.52 and 0.48 observed in the present study. The findings of Jang *et al.* (2005) corroborated with the earlier findings of Chung *et al.* (1998) wherein they have also reported the similar frequencies with respect to AA (0.621) and AB (0.218) genotypes as well as A (0.730) and B allele (0.270). Surprisingly, none of the animals from these studies (Jang *et al.* 2005, Chung *et al.* 1998) exhibited homozygous BB genotype. Also in a study by Dybus *et al.* (2005), the two herds of Black and White Polish cattle presented results contrary to the findings of the present study, wherein AA genotypes was observed with highest frequency (0.7222 and 0.6983) followed by the heterozygous AB genotypes (0.2778 and 0.2931) and BB genotype (0 and 0.0086), which was least represented. Same study however revealed quite a different picture for Jersey cattle, where BB genotype was most frequent (0.4757) followed by AA and AB.

*PRL-Rsa*I locus has been observed to have a significant effect on milk yield and fat percentage in dairy cattle (Chung *et al.* 1998), with the findings that cows with the AA genotypes of the *PRL* gene had higher milk protein content than AB individuals. In contrast a non-significant or weak association between *Rsa*I locus polymorphism in *PRL* gene and milk production traits was found for Black and White cattle and also for Jersey cattle (Dybu s*et al.* 2005). Considering these findings, usefulness of *PRL-RsaI* polymorphism as one of the candidate marker for the improvement of production traits of Ladakhi cattle needs critical evaluation.

Polymorphism at butyrophylin (*BTN1&3*) *genes:* Several polymorphisms of BTN genes (Taylor *et al.* 1996, Husaini *et al.* 1999) as well as their impact on production traits (Komisarek *et al.* 2003, Komisarek *et al.* 2006) has been reported. The PCR-RFLP analysis of 501 bp product of 7th exonic region of Butyrophylin 1 (*BTN1*) gene yielded significantly higher frequency of A (0.85) as compared to B allele (0.15) (Table 3) in Ladakhi cattle. The AA genotype was present predominantly followed by AB genotype (Fig. 2). None of the animal revealed BB genotype. This is in accordance with the allelic spectra observed for diverse cattle breeds (Fig.3). Taylor *et al.* (1996) reported the estimated gene frequencies of *BTN1* A and B alleles as 0.875 and 0.125, respectively. Husani *et al.* (1999) reported similar frequency level for allele A (0.850) and B (0.150). The trend of genotypic profile obtained in the present study also corroborates with the earlier study for Korean bulls, where very high prevalence of AA has been reported (Lee *et al.* 2002, Jang *et al.* 2005).

PCR-RFLP analysis for *BTN3/TaqI* locus revealed five genotypes AA, AB, BB, AC and AE genotypes (Fig. 1) with genotype frequencies 0.42, 0.31, 0.08, 0.15 and 0.04, respectively (Table 3). In addition, two new genotypes AC and AE were found which are novel to Ladakhi cattle and have not been observed in Indian native or taurine cattle. Allelic frequencies for A, B, C and E allele in Ladakhi cattle were 0.67, 0.23, 0.08 and 0.02 respectively. Similar to Ladakhi cattle, other Indian cattle breeds also showed higher frequency (0.71) of *BTN3/TaqI* A allele (Fig. 3). This particular variation could be of significance for Ladakhi cattle as this has been previously reported to be associated with production traits (Jiang *et al*. 2005).

Polymorphism at acyl CoA:diacyl glycerol acyltransferase (*DGAT1*) *gene*: CfrI based PCR-RFLP diagnostic test to differentiate allelic variants of K232A mutation in exon VIII (at nucleotide positions 10433–10434) of bovine diacylglycerol acyltransferase (*DGAT*1) gene revealed PCR amplified product of 411 bp length. *Cfr*I cleavage yielded two genotypes (Fig.2), KK (absence of any digestion; 411bp fragment) and KA (411, 208 & 203b fragments). The frequency of KK and KA genotype was 0.59 and 0.41 respectively (Table 3). This observation is in contrast to frequency spectra of other Indian native cattle breeds where only KK genotype has been observed (Tantia *et al*. 2006) and *DGAT*1K allele is fixed (Fig.3). Exceptionally high frequency of *DGAT*1K allele was found in Gyr and Red Sindhi Brazilian *Bos indicus* cattle (Lacorte *et al.* 2006) and extremely low frequency in European *B. taurus* breeds (Ripoli *et al.* 2006). Complete fixation of *DGAT1K* allele was observed in Brazilian Nellore and Guzerat zebu cattle, as all the animals of these two breeds were homozygous for the K allele which has the origin in Indian subcontinent. However, the Brazilian crossbred samples, viz. Gyr × Holstein, Nellore × Angus exhibited relatively high A allele frequency of 0.39 and 0.44, respectively, which might have attributed to their cross breeding with taurine populations (Pappas *et al.* 2004, Lacorte *et al.* 2006) High prevalence of DGAT1A allele has also been reported (Kaupe *et al.* 2004) for several taurine cattle breeds, viz. Brazilian Holstein (0.73), New Zealand Holstein (0.40), German Holstein (0.42), New

Zealand Jersey (0.88) and German Brown Swiss (0.98). The association of K232A with milk fat and protein yield has been well documented in Dutch Holstein dairy cattle (Grisart *et al.* 2002), New Zealand Holstein dairy cattle population (Spelman *et al.* 2002) and in German Holstein dairy cattle (Thaller *et al.* 2003). All these authors reported a strong allele substitution effect indicating *DGAT*1, as major gene influencing milk composition and production traits. Higher value for *DGAT*1*K* (0.79) is indicative of high fat percentage in Ladakhi cattle.

Considering the fact that Ladakhi cattle is a unique germplasm having excellent adaptation potential to high altitude hypobaric stress and resource for A2 milk, it is important to generate awareness amongst the policy planners, field functionaries, farmers and all stakeholders of the region about the importance of this precious germplasm. Further, local farmers and stake holders should be educated to maintain the purity of this population and avoid mixing of local cattle with exotic Jersey cattle. With the implementation of suitable breeding and management strategies, ladakhi cattle has the potential to be established as a lifeline for Ladakh region.

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