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Identification of the CSN1S1 allele in Indian goats by the PCR-RFLP method

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The allelic distributions of the CSN1S1 (αs_1-casein) in the Indian goats are quite different from European goat breeds. Majority of Indian goat breeds and non-descript goats carry A, B, E and F alleles at αs_1-casein locus, as found by analysing both DNA and protein levels. However, A and B alleles, known to be associated with better casein yield, were observed in the highest proportion in all the Indian goat breeds. Gene frequency and breed heterozygosity were computed for the CSN1S1 gene. The gene frequency of allele A in Indian goats varies from 0.68 to 1.00 and allele B varies from 0.098 to 0.23. Allele F was observed in Beetal, Marwari, Chegu and non-descript goats of MP (Local MP) in less than 1% of population. The expected heterozygosity (He) varied from 0.141 to 0.506 over the population. The Beetal breed showed the highest gene diversity (0.506) followed by Jamunapari (0.395), Chegu (0.383) and Jakhrana (0.381) breeds. Therefore, the variability at CSN1S1 locus can be utilised for conservation as well as for genetic improvement of Indian goat breeds for increasing both the quality and quantity of milk production.

Keywords: casein, genetic diversity, goats, polymorphism, RFLP

Introduction

Casein, the main protein fraction of ruminant milk, is one of the most valuable components due to its nutritional value and processing properties. The casein fraction is encoded by four tightly linked genes and is organised as a cluster in a 250-kb genomic DNA segment in the following order: αs_2-casein (CSN1S2), αs_1-casein (CSN1S1), β-casein (CSN2) and κ-casein (CSN3) (Grosclaude *et al*., 1987; Ferretti *et al*., 1990; Threadgill and Womack, 1990). They have been mapped on chromosome 6 in cattle and goats (Hayes *et al*., 1993; Popescu *et al*., 1996). The existence of extensive polymorphism at αs_1-casein provided unusual quantitative and qualitative differences in casein synthesis (Boulanger *et al*., 1984; Grosclaude *et al*., 1987; Ramunno *et al*., 1991) as well as its importance to the application of milk protein research to dairy industry (Bevilacqua *et al*., 2002; Feligini *et al*., 2005). The αs_1-casein, which has known 16 co-dominant alleles, is associated with different rates of protein synthesis. It has been established that the A, B and C alleles at CSN1S1 locus associated with the production of a high level of protein in milk, the E allele associated with a medium-level protein and F and G alleles with a low-level protein (Grosclaude *et al*., 1987 and 1994). The distribution of different alleles at CSN1S1 locus has been investigated in European countries at the genomic as well as protein levels (Grosclaude *et al*., 1994, Ramunno *et al*., 1991 and 2000; Bevilacqua *et al*., 2002; Sacchi *et al*., 2005). However, casein variability has not been well characterised in Indian goats except for the reports of Prakash *et al.* (2002) and Rout *et al*. (2004a). Moreover, milk protein gene diversity in cattle showed a strong relationship with human lactase gene and has been used to analyse geographical and genetic diversity in European cattle (Beja-Pereira *et al*., 2003). India has a high goat population, which is distributed over all the regions of the country, and produce significant amount of goat milk. The characterisation of CSN1S1 casein variability is important due to its relationship with cheese production and milk-processing properties. Therefore it is important to evaluate the variability of αs_1-casein gene in different Indian goat breeds and to determine the effect of αs_1-casein genotyping for genetic improvement as well as for a conservation programme. Therefore the present study has been designed to characterise αs_1-casein (CSN1S1 locus) in Indian goats by both genomics and protein level.

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Material and methods

Sample collection and DNA isolation

A total of 347 unrelated blood samples were collected from goats in their natural habitats belonging to the four major geographical agro-climatic zones of India, including at least one breed from each major geographical region. An effort was made to collect samples from unrelated individuals based on the information provided by farmers. DNA was isolated from the samples using the standard protocol published elsewhere (Thangaraj *et al*., 2002).

We analysed 13 different genetic groups from various agro-climatic zones for CSN1S1 gene variability by DNA analysis (Table 1). As we need to correlate structural variation with functional variation in different goat breeds, genotyping was carried out to analyse milk protein variability at CSN1S1 locus. Therefore, we collected milk samples from all major milk-producing goat breeds namely Jamunapari, Barbari, Marwari, Sirohi, Jakhrana and Beetal belonging to the arid and semi-arid climatic zones, and from two non-descript goats (Local Madhya Pradesh (MP) and Local Uttar Pradesh (UP)) available in the same agro-climatic area (Table 2). Milk protein analysis was carried out to provide supporting evidence on variation at the DNA level.

Milk samples were collected from 1058 goats of Jamunapari, Barbari, Marwari, Sirohi, Jakhrana, Beetal, Local UP and Local MP goats from the natural habitat of each breed and transported to the laboratory and stored at −20°C for further analysis.

Genetic stocks

Jamunapari goats are found in isolated pockets and sampling was carried out in their natural habitat in the Chakarnagar area of Etawah district of UP. The breed is known as the best Indian dairy goat (Rout *et al*., 2004b) and the breed risk status was classified as endangered (http://dad.fao.org) (Rout *et al*., 2000). Similarly, Jakhrana is known for high milk yield from the Alwar area of Rajasthan. Goats are distributed in few villages in their home tract and the number is also decreasing in the natural habitat. Barbari is a medium-sized breed of the semi-arid zone and known for its adaptability over a wide range of agro-climatic situations. Beetal is one of the largest breeds of goat and reared mainly for milk but is equally important for meat. This breed is found in Punjab along the Indo-Pakistan border and the number is decreasing in the region. Sirohi is a medium- to large-sized breed and is best known for meat, milk production and resistance to a number of diseases. The natural habitat of the breed is in the wider area of Ajmer, Bhilwara, Tonk and Jaipur in Rajasthan. Marwari is a medium-sized breed with a compact body and strong legs and is known for its hardiness and adaptability to extreme temperatures. Marwari is also known for meat, milk and coarse fibre production. The Black Bengal is the typical dwarf breed of eastern India and known for its high prolificacy and meat quality. Osmanabadi goats are a medium-sized breed with a comparatively long body and long legs and are found in the Ahmednagar and Solapur area of Maharashtra. Gaddi goats are adapted to a hilly environment, found in the hills of Himachal Pradesh and are known for their draftability. Chegu goats are known for Pashmina production and are found at high altitudes of Lahaul and Spliti Valley of Himachal Pradesh, Uttarkashi, Chamoli and Pithoragarh district of Uttaranchal. Surti goats are found predominantly in the Bhavnagar area of Gujarat and is known for its milk production. 'Local' goats are non-descript goats found in the area adjacent to Central Institute for Research on Goats (CIRG), Makhdoom and Hathras area of UP, designated as Local UP, and from adjacent region of Gwalior and Morena area of MP, designated as Local MP. These goats are mainly dual-purpose goats and reared by local farmers.

Genotyping

The variation at the DNA level was analysed in 13 genetic groups (Table 1). Milk protein variation was measured in seven populations to provide supporting evidence for genomic variability (see Table 2). DNA samples were analysed with allele-specific polymerase chain reaction (AS-PCR) and the amplified product was digested with *XmnI* for the 13 genetic groups.PCR was carried out in a 50 μl reaction mixture containing 100 ng genomic DNA, 10 pmol of each primer (forward: F 5′TTCTAAAAGTCTCA GAGGCAG-3′, reverse: 5′ GGGTTGATAGCCTTGTATGT 3′), 1.25 U of TaqDNA polymerase, 50 mmol/l KCl, 10 mmol/l Tris–HCl (pH 9.0), 0.1% Triton X-100, 3 mmol/l $MgCl_2$, dNTPs each at 400 μmol/l, 0.04% BSA (Ramunno *et al*., 2000). The amplification protocol was used as follows: an initial cycle of 97°C for 2 min, 60°C for 45 s and 72°C for 2 min 30 s; then 30 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 2 min 30 s and a final extension step 72°C for 10 min. Restriction analysis was carried out using *XmnI* enzyme. For this, 20 μl of each PCR product was digested with 10 U of *XmnI* endonuclease for overnight at 37°C and digested products were analysed in 4% agarose gel stained with ethidium bromide and analysed in a gel documentation system (Alpha Innotech Corporation, San Leandro, CA, USA).

SDS-PAGE was carried out in skimmed milk samples and was analysed by means of SDS (Grosclaude and Martin, 1997) and urea (Medrano and Sharrow, 1989) PAGE and alkaline pH. Gels were stained with Coomassie Brilliant Blue. Milk protein variants were determined by the molecular weight in gel documentation system (Alpha Innotech Corporation).

Statistical analysis

Genepop (Raymond and Rousset, 1995) software was used to estimate allelic frequencies, expected heterozygosity, effective number of alleles and to verify Hardy–Weinberg equilibrium. The genotypes were observed by counting the patterns in the gel documentation system. Variance and

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Table 1 *Genotypic and allelic frequencies with 95% confidence interval (CI), effective number of allele and expected heterozygosity after* XmnI *digestion of fragment obtained from PCR of the DNA region spanning from eighth to the ninth intron of goat αs1-casein locus Indian goat populations*

		Genotypic frequency				Allelic frequency						
Breed	No. of animals	AA	BB	AD	AF	A	B	D	F	95% CI of allele frequency	Effective no. of allele	Expected heterozygosity
Jamunapari	35	0.714	0.229	0.057	–	0.743	0.228	0.029	–	0.142	1.653	0.395
Barbari	35	0.857	0.114	0.029	–	0.871	0.114	0.015	–	0.109	1.294	0.227
Marwari	35	0.743	0.142	–	0.115	0.800	0.143	–	0.057	0.122	1.506	0.336
Sirohi	17	0.764	0.118	0.118	–	0.823	0.118	0.059	–	0.165	1.437	0.304
Jakhrana	42	0.714	0.190	0.096	–	0.762	0.190	0.048	–	0.122	1.615	0.381
Beetal	20	0.500	0.150	0.200	0.150	0.675	0.150	0.100	0.075	0.162	2.02	0.506
Chegu	26	0.731	0.231	–	0.038	0.750	0.230	–	0.020	0.165	1.62	0.383
Local UP	20	1.000	–	–	–	1.000	–	–	–	–	1.00	–
Local MP	20	0.900	0.050	–	0.050	0.925	0.050	0.000	0.025	0.106	1.164	0.141
Black Bengal	36	0.810	0.190	–	–	0.805	0.195	–	–	0.132	1.45	0.313
Gaddi	21	1.000	–	–	–	1.000	–	–	–	–	1.00	–
Surti	21	0.857	0.143	–	–	0.857	0.143	–	–	0.153	1.324	0.244
Osmanabadi	51	0.745	0.098	0.157	–	0.824	0.098	0.078	–	0.077	1.44	0.306

95% confidence interval were calculated according to the formula (Var $(p_u) = \frac{1}{2}N(p_u + P_{uu} - 2p_u^2)$) suggested by Weir (1996) (P_{uu} = genotypic frequency; p_u = allelic frequency).

Results

DNA samples were analysed for the presence of different αs_1-casein allele by single-AS-PCR. The region of goat CSN1S1 gene between nucleotide 208 and 420 spanning part of eighth intron, ninth exon and part of the ninth intron was amplified and digested with *XmnI*. Observed genotypic and allelic frequency at the CSN1S1 locus of different Indian goat breeds are presented in Table 1.

Genotyping of 347 individuals belonging to 13 different genetic groups or breeds of Indian goats was carried out with the PCR-RFLP method. The PCR amplified product was observed as 223 bp (Figure 1). Four different variant groups of (150 + 63) bp, (161 + 63) bp, (212 + 150 + 63) bp and (223 + 150 + 63) bp were obtained (Figure 2) after restriction digestion with *XmnI*. Genotyping at the DNA level showed that the AA genotype had the highest frequency in Indian goats. Comparing results from both DNA and milk samples of individuals revealed the (150 + 63 bp) haplotype in higher proportion in all the breeds except in Local UP goats. This haplotype is associated with the presence of allele $CSN1S1^A$ and $CSN1S1^{01}$ alleles (null allele) at CSN1S1 locus. The percentage of (161 + 63 bp) haplotype varies from 5% to 22.9% in the Indian goat population, indicating a significant proportion of B and E alleles in the population. The electrophoretic pattern in SDS-PAGE did not show the presence of the E variant in the goat breeds. AF genotypes (223 + 150 + 63 haplotype) were observed in Marwari, Beetal, Chegu and Local MP goats indicating the presence of F and A alleles in the population. The F variant was also observed in SDS-PAGE, which was confirmed by the presence of the F allele in Marwari, Beetal, Chegu and Local MP goats. The highest percentage of (212 + 50 + 163bp) haplotype was observed in Beetal, Osmanabadi and Sirohi goats, indicating the presence of the A and D allele in the population and the D allele was not observed in SDS-PAGE. The presence of 11 bp insertion (223 or 224 bp) was also observed in the Indian goats. The absence of 11 bp insertion (212 or 213 bp) was also observed in the Indian goats at the ninth exon. The Indian goats showed the presence of A, B, E and F alleles at αs_1-casein locus by both DNA and protein analysis.

Milk protein analysis of SDS-PAGE pattern revealed a similar type of variability at CSN1S1 locus in the Indian goats and confirmed the findings as obtained from DNA analysis. The αs_1-casein A allele was observed in the majority of goats and their frequency in Jamunapari, Barbari, Marwari, Sirohi, Jakhrana, Beetal, Local UP and Local MP was 0.71, 0.77, 0.56, 0.76, 0.67, 0.72, 0.58 and 0.52, respectively. The B variant was observed as heterozygous

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Table 2 *Gene frequency of different milk protein variants at CSN1S1 locus in nine different genetic groups by SDS-PAGE*

Breeds	No. of animals	Allele			
		αs1-cn^A	αs1-cn^B	αs1-cn^F	αs1-cn^O
Jamunapari	179	0.718	0.006	0.149	0.127
Barbari	475	0.774	0.011	0.122	0.093
Marwari	70	0.564	0.014	0.414	0.008
Sirohi	69	0.768	0.065	0.036	0.130
Jakhrana	68	0.677	0.000	0.176	0.147
Beetal	45	0.722	0.000	0.167	0.111
Local UP	110	0.586	0.000	0.186	0.228
Local MP	42	0.525	0.000	0.075	0.400

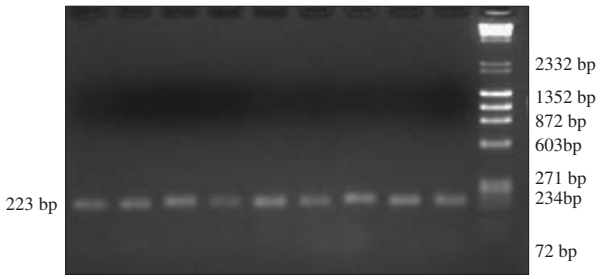

Figure 1 PCR amplification of DNA region spanning from eighth to the ninth intron of the goat αs1-casein gene.

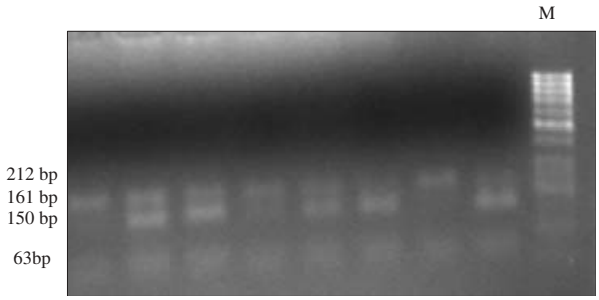

Figure 2 Observed *XmnI*-RFLP genotype obtained by PCR amplification of the DNA region spanning from eighth to the ninth intron of goat αs1-casein gene, M = marker (50 bp DNA ladder).

AB in the Indian goat breeds .The F allele was distributed as heterozygous AF in different goat breeds. (Table 1)

Gene frequencies and their 95% confidence interval were presented in all the studied populations (13 genetic groups; Table1). Hardy–Weinberg equilibrium (HWE) was tested by χ^2-tests in popgene software and there was no significant departure from Hardy–Weinberg equilibrium ($\chi^2 >= 24.65$) observed in the analysed population. The expected heterozygosity (He) varies from 0.141 to 0.506 over the population. The Beetal breed showed highest gene diversity (0.506) followed by Jamunapari (0.395), Chegu (0.383) and Jakhrana (0.381; Table 1). Barbari, Surti and Local MP goats showed lower heterozygosity as compared with other breeds. Similarly, the effective number of alleles varied from 1.00 to 2.02 over the genetic groups. The effective number of allele was highest in Beetal (2.02) followed by Jamunapari, Chegu and Jakhrana (Table 1). No variability was observed at CSN1S1 locus in Gaddi and Local UP samples. Beetal, Jamunapari and Jakhrana breeds showed higher gene diversity and effective number of allele at CSN1S1 locus and are considered as threatened breeds in their natural habitat.

Discussion

CSN1S1 is characterised by 19 exons ranging in size from 24 (exons 5, 6, 7, 8, 10, 13, 16) to 17.5 kb (Jansa Perez *et al*., 1994). The goat CSN1S1 locus has been characterised by at least 13 alleles, which have been associated with different levels of protein synthesis (Grosclaude *et al*., 1987; Martin, 1993). Grosclaude *et al*. (1987) reported that the amount of total casein in caprine milk was positively correlated with the presence of αs_1-casein allele and was highest in case of A, B and C alleles. (Boulanger *et al*., 1984; Grosclaude *et al*., 1994; Roncada *et al*., 2002).

Molecular analysis at the DNA level showed A, B, E and F alleles at αs_1-casein locus, and A and B alleles were observed in highest proportion in the Indian goat breeds. SDS-PAGE analysis also indicated that the Indian goat breeds are carrying the A allele in higher frequency. Local UP, MP, Gaddi, Black Bengal, Surti, Sirohi and Barbari breeds showed a very high frequency allele A (Table 1). The Beetal breed showed the lowest allele A frequency at this locus, but taking into consideration the 95% confidence interval the frequency varied from 0.513 to 0.837, which is not different from other breeds. However, the sample size of Beetal, Surti, Sirohi and Local MP breeds was less than 25. There is about a 95% chance that the interval included population frequency provided the sample is reasonably large ($n > 30$) (Weir, 1996; Lewis *et al*., 2004). The frequency of F and E alleles was low in the Indian goats. Allele D was observed in heterozygous forms in the Indian goat breeds by DNA analysis and not observed in SDS-PAGE, which needs further characterisation. Molecular analysis showed the presence of A, B, E and F alleles in the Indian goats and the same was confirmed by SDS-PAGE analysis. SDS-PAGE revealed a lower number allele as it was not possible to determine all variants at the protein level as different variants co-migrate with each other (Grosclaude *et al*., 1994).

Indian goats are better producers of milk as well as protein in comparison with goat breeds of the other regions. The Italian goat breeds, Garganica and Maltase, exhibited αs_1-cn^A frequency as 0.61 and 0.33, respectively, and the Spanish goats, Palmera and Canaria breeds, showed αs_1-cn^A frequency as 0.68 and 0.28, respectively (Grosclaude *et al*., 1987; Ramunno *et al*., 1991; Jordana *et al*., 1996). The Spanish goats, Maurciano-Granadina,

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Malaguena, Payoya and Majorera, showed very low αs_1-cn^A frequency (0.05 to 0.28) and higher frequency of E and B alleles (Grosclaude *et al*., 1987). Alpine and Saanen goats from France showed αs_1-cn^E and αs_1-cn^F allele frequencies as 0.34 and 0.41, respectively, and αs_1-cn^A frequency as 0.14 and 0.07, respectively (Grosclaude *et al*., 1987 and 1994). Togenburg, Appenzeller and Verzasca breeds of Switzerland had αs_1-cn^A frequency as 0.01 for all the breeds and αs_1-cn^F frequency was 0.69, 0.44 and 0.62, respectively (Grosclaude *et al*., 1987 and 1994). The frequency of α_{S1}–cn^F locus was lower in Spanish breeds (0.08, 0.04, 0.0 and 0.0 for Murciano-Granadina, Malaguena, Payoya and Canaria, respectively) while the E allele was predominant in Murciano-Granadina (0.59), Malaguena (0.65) and Payaya (0.76) breeds (Jordana *et al*., 1996).

Although Alpine and Sannen produce large amounts of milk, protein content is less as they carry a defective allele in the genome (Grosclaude *et al*., 1987). The Indian breeds show A and B alleles in higher frequency indicating the better allelic combination for the higher protein yield in comparison with other breeds reported in different parts of the world. Grosclaude *et al*. (1994), based on sequence comparison of CSN1S1 gene between the different ruminant species, concluded that high alleles represent the ancestral sequences of this gene in goats and the frequency of high allele in Indian goats observed both in established breeds and non-descript goats. As a significant proportion of Indian goats are showing distribution of E and $CSN1S1^{01}$, alleles at DNA level therefore need further characterisation.

Jamunapari, Beetal and Jakhrana breeds exhibited higher gene diversity and effective number of alleles and are presently considered as threatened breeds in their respective home tract. The number of animals is decreasing due to the change in agricultural practices and for other reasons. The analysis indicated there must be conservation of these breeds with respect to milk protein variability. Milk protein diversity showed Beetal as a unique breed indicating the need to conserve the germplasm as the breed is facing extinction in its home tract for several reasons. Moreover, milk protein gene diversity has been analysed in order to explain the cultural evolution of the lactase gene between humans and bovines and also for use in establishing geographical diversity and conservation decisions (Beja-Pereira *et al*., 2003). Indian goat breeds are showing considerable variability at the CSN1S1 gene in different geographical areas, and this needs further study to establish the relationship between the utility of goat germplasm in relation to human food, habit and migration. Analysis of geographical and molecular diversity along with food and habit (including other cultural diversity) will establish a new horizon for conserving the goat breeds.

The effects of αs_1-casein polymorphism on milk yield and composition, micelle structure, renneting properties and cheese yield have been thoroughly studied in French breeds (Remeuf, 1993; Grosclaude *et al*, 1994; Mahé *et al*., 1994; Vassal *et al*., 1994; Barbieri *et al*., 1995; Ricordeau *et al*., 1996; Martin *et al*., 1999; Ricordeau *et al*., 2000). There is evidence that goats associated with the high content (A or B allele) of αs_1-casein produce milk characterised by a significantly high percentage of protein, fat, total calcium, better curd-firming time, curd firmness and cheese yield compared with goats homozygous for alleles associated with a low or intermediate content (E or F allele). However, in our earlier reports, we had observed that αs_1-casein genotype had a significant effect on protein content and calcium content in Jamunapari, Barbari, Marwari, Jakhrana and Sirohi goat breeds (Prakash *et al*., 2002; Rout *et al*., 2004a). The benefit from using the information on αs_1-casein genotype in a selection programme for dairy goats will improve the protein content (Sanchez *et al*., 2005). The present study and previous reports on Indian goats also provide a clear indication that protein content can be improved by selecting the αs_1-casein genotype. Therefore, genotyping at CSN1S1 locus should be carried out for better cheese yield and a genetic improvement programme.

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