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An investigation on allele frequency at the *CSN1S2* locus and its relationship with milk parameters in the Sarda goat*

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ABSTRACT

The aim of the study was to assess allele frequencies at the *CSN1S2* locus in the Sarda goat and the effects of the genotype on milk composition. Two hundred twenty Sarda goats from 20 farms were selected. Individual blood and milk samples were collected during the middle of lactation and daily milk yield was registered. Fat, protein and lactose percentage, freezing point, pH, somatic cell count and total mesophilic count were measured. DNA was analysed with different methods based on PCR. Allele frequencies, the Hardy Weinberg (HW) equilibrium and the correlations between milk yield and composition and the genotypes were calculated. F (0.400) and A (0.330) alleles showed the highest frequency. D and 0 alleles were not found. Genotype frequencies were the following: AA, 0.136; AB, 0.009; AC, 0.082; AE, 0.032; AF, 0.264; CC, 0.023; CE, 0.023; CF, 0.250; EF, 0.077; FF, 0.105. The population was in HW disequilibrium. No link between the genotypes and milk yield, chemical, physical and cytological parameters was found.

KEYWORDS: *CSN1S2* locus, milk, goat

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INTRODUCTION

Breeding and the improvement of autochthonous breeds is an effective choice to improve animal husbandry in some developing areas. Natural and typical products are also guaranteed by the preservation of biodiversity in livestock species and their breeding habitat (Boyazoglu et al., 2005). The Sarda is the autochthonous goat from the island of Sardinia and, with about 200,000 heads, it is considered a large reservoir of biodiversity. Animal products from the Sarda goat could safeguard this breed and the whole goat breeding activity, which plays an important economic and social role in Sardinia (Carcangiu et al., 2006). Sarda selection schemes have still not considered the qualitative traits of the milk, such as the genetic characterization of casein variants, which are correlated with the chemical and technological properties of goat milk (Barbieri et al., 1995). The study of these characters in other species and breeds has allowed the enhancement in value of their products. The αs_1, αs_2, β and κ-caseins are encoded by four strictly associated genes. In the bovine species, they are located on chromosome 6, within a 250 Kbp DNA segment, in the following order: *CSN1S1* (encoding αs_1-casein), *CSN2* (β-casein), *CSN1S2* (αs_2-casein), *CSN3* (κ-casein) (Threadgill and Womack, 1990). The organization of the casein gene cluster is highly conserved among mammals, although high qualitative and quantitative variability have been recorded between the species (Rijnkels, 2002). Several studies evidenced that the four goat casein genes are polymorphic (Caroli et al., 2006). This variability is an important factor in genetic improvement and permits a diversification of milk production, thus allowing choice for the right genetic basis for the production of cheese, drinking or hypoallergenic milk (Ramunno et al., 2007). αs_2-casein is a phosphoprotein of 208 amino acids and is one of the most important allergens in milk (Jarvinen, 2002). El-Agamy (2007) stated the risk of allergy reaction is diminished as regards goat milk as its milk proteins show a high homology with human ones. Moreover, αs_2-casein is absent in human milk (Martin et al., 1996), although two potential αs_2-like casein genes have been identified within the human casein gene cluster, named *CSN1S2A* and *CSN1S2B*. The DNA transcript of *CSN1S2A* has been detected in the human lactating mammary gland RNA, but it probably does not produce a stable protein chain. Specific DNA transcripts of *CSN1S2B* have not been found (Rijnkels et al., 2003). In cattle, *CSN1S2* is the longest casein gene (about 18,5 Kbp) and it is organized in 18 exons, whose length varies between 21 and 266 bp (Groenen et al., 1993). In goats, seven alleles have been identified at the *CSN1S2* locus and these are associated with three different levels of αs_2-casein in milk. A, B, C, E and F "strong" alleles are associated with a normal αs_2-casein content (about 2.5 g/l for allele), D allele with an intermediate content (1.5 g/l) and the "null" allele 0 in homozygosis is associated with the

apparent absence of αs_2-casein in milk (Ramunno et al., 2001).

The aim of this research was to analyse the genetic structure at the *CSN1S2* locus in Sarda goats and the possible correlations between its genotype and milk parameters.

MATERIAL AND METHODS

Animals and samples

A total of 220 lactating goats from 20 goat farms (11 goats in each farm) located in central Sardinia (Italy), were randomly chosen. Farms were similar in animal management which was in accordance with traditional Sardinian goat farming: goats were exclusively fed pasture on a Mediterranean shrubland, without any concentrate supplementation and the pasture composition was similar among the farms; they were hand-milked once daily in the morning and in all the flocks the udder was not cleaned before milking; reproduction was based on natural mating and sex ratio was 1/18; kids were milk fed by their dams and weaned when they were about six weeks old; some pens and a shelter for milking were the only facilities on the farms. During the intermediate stage of lactation (between day 100 and 110 after kidding) individual milk samples were collected from all goats in 200 ml sterile plastic containers and were transported at +4°C to the laboratory within 2 h. Plastic containers were filled after the first stream of milk had already been rejected. On the same day, individual daily milk yield in grams was registered and a blood sample was taken by one puncture from the jugular vein of each animal, using vacuum tubes with EDTA as anticoagulant (BD Vacutainer Systems®, Belliver Industrial Estate, Plymouth, UK).

Milk analysis

Milk samples were analysed for pH and total protein, fat, lactose and urea content using an I.R. spectrophotometer (Milko-Scan 133B Foss Electric®, DK-3400 Hillerød, Denmark) according to the International Dairy Federation (IDF) standard (IDF 141C:2000); somatic cell count (SCC) using an automatic cell counter (Fossomatic 90, Foss Electric®) according to IDF 148A:1995; total mesophilic count (TMC) using a Bacto-Scan, Foss Electric® (IDF 358:2000); freezing point (FP) in Hortvet degrees (H°) by a thermistore cryoscope (IDF 108:2002).

PCR amplification and polymorphism identification

The DNA was extracted from leukocytes using the Puregene® DNA isolation kit (GENTRA) and analysed by means of different PCR methods. The *CSN1S2* B allele, compared to the A allele, shows a G10A transition at exon 9, which causes a Glu_{64}→Lys substitution in the mature protein, while the mutational event characterizing the C variant is a transversion A5T at exon 16, which determines the substitution Lys_{167}→Ile (Bouniol et al., 1994). The presence of *CSN1S2* A, B and C alleles was investigated by Multiplex Allele Specific PCR (Ramunno et al., 2000), utilizing in the first reaction the two primer pairs: B1X/B1Z and C2X/C2Z, which selectively amplified the *CSN1S2* A allele, and in the second PCR reaction the two primer pairs: B1Y/B1Z and C2Y/C2Z, which amplified the CSN1S2 B and C alleles, respectively. The *CSN1S2* D and 0 alleles were investigated by PCR-RFLP (Ramunno et al., 2001). The *CSN1S2* D allele is characterized by the deletion of 106 bp spanning from the 11th nt of exon 11 to 95 nt of the following intron. This allele was detected by PCR amplification of a 301 bp long fragment containing the 11th exon, with the primer pair CASDF/CASDR; the D allele, if present, produces a fragment of 195 bp. The same PCR amplified fragment allowed the detection of the *CSN1S2* 0 allele, after digestion with the restriction enzyme NcoI, being the 0 allele characterized by a transition G80A at exon 11, which causes the formation of a premature stop codon. The nucleotide variation characterizing the *CSN1S2* E allele (C83G) occurs at exon 16 and results in an amino acid substitution Pro_{193}→Arg in the mature protein. It was detected by PCR amplification (primers CASEF/CASER) and restriction with enzyme NlaIII (Veltri et al., 2000). The *CSN1S2* F allele is characterized by a G13C transition occurring at exon 3, which results in an amino acid substitution Val_{7}→Ile. This variant was detected by PCR amplification (primer pair CASFF/CASFR) and digestion with the restriction enzyme BsmaI (Ramunno et al., 2001). Primer sequences are reported in Table 1. All the PCR amplification protocols were performed in a reaction mixture of 25 µl final volume, where 100 ng genomic DNA was amplified with 0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1x reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl) and 1U Taq Polymerase (Platinum® Taq DNA Polymerase, Invitrogen, Italy). PCR was performed on a Mastercycler® ep gradient S Thermal Cycler (Eppendorf, Italy), and consisted of an initial denaturation step at 94°C for 2.5 min, followed by 30 cycles at 94°C for 20 sec, the appropriate annealing temperatures for each primer pair (Table 1) for 30 sec, and 72°C for 30 sec, with a final extension step at 72°C for 10 min. The amplification products were visualized by agarose gel electrophoresis and ethidium bromide staining.

Table 1. Primer sequence, product size and annealing temperature used for *CSN1S2* genotyping

Primer name	Forward primer sequences 5'- 3'	Primer name	Reverse primer sequences 5'- 3'	Product size, bp	Annealing temp. °C
B1Z	CTATCAGATCATCTAGTGAG	B1X	CTCTGGGGCAACTTC	1085	53
C2Z	CTGAAGAAGAAAAGAATCGCC	C2X	CTGGTAATACTGGCTGATTT	808	53
CASDF	GACACATAGAGAAGATTC	CASDR	CGTTGGGACATTTTATCT	301/195	45
CASEF	GGTTAGGTCTAGGTGTTCTGA	CASER	TTTTTATTACAAAAGACAACT	232	47
CASFF	TCTCTTGCCATCAAAACA	CASFR	TGGTCTTTATTCCTCTCT	310	50

Statistical analysis

Allele and genotype frequencies and Hardy-Weinberg equilibrium were calculated using Genepop software (Raymond and Rousset, 1995). Analysis of variance was performed using Minitab statistical software, Minitab release 13.32 (Minitab Inc. 2000, State College, PA) in order to evidence the effect of the different μs_2-casein genotypes on milk yield and composition. The model used for all variables was:

$$Y_{ij} = \mu + F_i + G_j + ek_{(ij)}$$

where: Y_{ij} - each milk parameter, μ - general mean, F_i - the fixed effect of flock (i=20), G_j - the random effect of each individual goat (j=220), $ek_{(ij)}$ - error effect.

For all parameters, model effects were declared significant at $P<0.05$. Prior to this, SCC and TMC had been transformed into logarithmic form to normalize the distribution of their frequencies (Snedecor and Cochran, 1980).

RESULTS

Allele and genotype frequencies at the *CSN1S2* locus are reported in Table 2. F and A alleles were the most prevalent (0.330 and 0.400, respectively), B showed the lowest frequency (0.005) as it was identified, in heterozygosis, in

Table 2. Allele and genotype frequencies at *CSN1S2* locus in the Sarda goat (n=220)

Allele	Frequency	Genotype	n	Frequency, %
A	0.330	AA	30	13.6
B	0.005	AB	2	0.9
C	0.200	AC	18	8.2
E	0.065	AE	7	3.2
F	0.400	AF	58	26.4
		CC	5	2.3
		CE	5	2.3
		CF	55	25.0
		EF	17	7.7
		FF	23	10.5

only two goats, while D and 0 alleles were not detected. On the whole, 10 different genotypes were identified. AF and CF, with a frequency of 26.4 and 25%, respectively, were the most common, as the sum of their frequencies represented more than 50% of the examined samples.

Table 3. Means (± SD) of milk yield and composition according to *CSN1S2* genotype in the Sarda goat (n=220)

CSN1S2		AA	AB	AC	AE	AF	CC	CE	CF	EF	FF	Mean
genotype	n	30	2	18	7	58	5	5	55	17	23	220
Milk yield, g/d	mean	826.4	1025.0	860.4	704.8	864.5	926.8	716.4	861.5	800.0	818.6	842.4
	±SD	472.0	534.1	347.4	370.9	400.4	363.5	249.0	396.1	339.9	277.2	381.6
Fat, %	mean	5.11	4.78	5.22	5.51	5.05	4.05	5.66	5.05	4.81	5.05	5.05
	±SD	0.89	0.40	1.39	1.18	1.28	1.58	1.57	1.42	1.29	1.06	1.26
Protein, %	mean	4.25	4.05	4.24	4.36	4.14	3.85	4.73	4.24	4.16	4.03	4.19
	±SD	0.61	0.16	0.68	0.44	0.58	0.97	0.54	0.63	0.60	0.51	0.60
Lactose, %	mean	4.89	4.62	4.90	4.87	4.91	5.01	4.88	4.93	4.73	4.84	4.89
	±SD	0.32	0.12	0.23	0.27	0.26	0.10	0.22	0.29	0.28	0.26	0.27
Freezing point, °H	mean	-0.569	-0.551	-0.569	-0.567	-0.568	-0.569	-0.565	-0.568	-0.570	-0.567	-0.568
	±SD	0.007	0.022	0.008	0.003	0.007	0.006	0.004	0.006	0.014	0.008	0.008
pH	mean	6.69	6.81	6.70	6.71	6.73	6.70	6.80	6.71	6.70	6.74	6.72
	±SD	0.07	0.13	0.08	0.08	0.07	0.06	0.04	0.09	0.09	0.07	0.08
Log SCC	mean	6.31	6.62	6.13	5.92	6.20	6.05	6.21	6.23	6.24	6.22	6.22
	±SD	5.56	5.75	5.62	5.18	5.64	5.54	5.59	5.95	5.99	5.92	6.37
Log TMC	mean	4.36	4.83	4.26	4.74	5.51	4.00	4.56	4.95	4.74	4.60	5.10
	±SD	4.08	4.32	4.04	4.23	5.17	3.48	4.18	4.58	4.32	4.34	5.06

Daily milk yield, chemical and physical composition, SCC and TMC according to genotype are summarized in Table 3. Statistical analysis did not evidence any difference among the genotypes. Average daily milk yield was 840 g, fat, average protein and lactose percentage were 5.05, 4.19 and 4.89%, respectively. Mean values of freezing point and pH were -0.568°H and 6.72. TMC logarithm was 5.10 and SCC logarithm 6.22.

DISCUSSION

Genotype frequencies of AF and CF were higher than those recorded by Ramunno et al. (2000) in a local goat breed reared in the south of Italy, in which AA genotype has the highest frequency (22.5%). Analysis of allele frequencies, in accordance with the Hardy-Weinberg law, indicated that the examined population was in disequilibrium because of a heterozygote excess. Marletta et al. (2005) also describe a disequilibrium at *CSN1S2* in Girgentana goats, but that is due to a heterozygote deficiency. The allele frequencies recorded for F, A and C were similar to those by Sacchi et al. (2005) in several Mediterranean goat breeds. Bouniol et al. (1994), in Alpine and Saanen goat breeds, register the highest frequency for the *CSN1S2* A allele (0.85) and the lowest for the C allele (0.11). In our study, allele frequencies of *CSN1S2* B and E were lower than those evidenced by Sacchi et al. (2005) in several Italian goat breeds. The defective D and 0 alleles were not identified in this study. Both alleles show a frequency lower than 0.05 in two studies by Sacchi et al. (2005) and Marletta et al. (2005) while a very high frequency of the 0 allele (0.15) has been reported in a population of Hungarian dairy goats (Kusza et al., 2007).

The polymorphisms occurring at a casein locus have to be considered in the contest of the casein gene cluster, using information deriving from the entire casein haplotype (Hayes et al., 2006; Finocchiaro et al., 2008). The goats analysed in this study showed the occurrence of defective alleles at the *CSN1S1* locus, coding for αs_1-casein, such as the F, E, 01 and N alleles, and also at the *CSN2* locus, coding for β-casein, such as the 01 allele (Vacca, unpublished). These variants were also detected in bucks from the Sarda breed (Vacca et al., 2005).

Daily milk yield was lower, but fat and protein content were higher than those recently registered by the Italian breeders Association (AIA) for specialized breeds reared in Italy, like Saanen and Alpine (http://www.aia.it/bollettino/bollettino.htm), and by several authors (Haenlein, 2007; Morand-Fehr et al., 2007). These results agree with previous studies on the Sarda goat (Tziboula-Clarke, 2003; Macciotta et al., 2005; Morand-Fehr et al., 2007) and are similar to those recorded by Galal (2005) for other non-specialized local goat breeds. TMC value, which is

considered an excellent parameter for assessing flock hygiene and health, was low, considering hand-milking and inadequate facilities on the farms. This statement is confirmed by the results regarding SCC values, which seem to be high if compared to other dairy species like cows or sheep, but are similar to those recorded in other goat breeds reared with extensive and intensive methods (Fekadu et al., 2005).

CONCLUSIONS

The present studies show that the αs_2-casein genotype did not affect any parameter and we can suppose this was due to the absence of the null and intermediate alleles. This feature indicates that the milk from Sarda goats is suitable for cheese making. Moreover, it could be interesting to continue the research in order to detect defective alleles in the Sarda breed, as goats carrying null and intermediate alleles will allow the production of drinking milk for people who are allergic or intolerant of cow's αs_2-casein.

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