Effect of *CSN1S1-CSN3* (α_{S1} - κ -casein) composite genotype on milk production traits and milk coagulation properties in Mediterranean water buffalo

V. Bonfatti,^{*1} M. Giantin,^{*} M. Gervaso,^{*} A. Coletta,[†] M. Dacasto,^{*} and P. Carnier^{*}

^{*}Department of Comparative Biomedicine and Food Science, University of Padova, viale dell'Università 16, 35020 Legnaro, Padova, Italy

[†]Associazione Nazionale Allevatori Specie Bufalina (ANASB), via C. Battisti 68, 81100 Caserta, Italy

ABSTRACT

The aim of this study was to estimate effects of *CSN1S1-CSN3* (α_{S1} - κ -casein) composite genotypes on milk production traits and milk coagulation properties (MCP) in Mediterranean water buffalo. Genotypes at *CSN1S1* and *CSN3* and coagulation properties [rennet clotting time (RCT), curd firming time (K_{20}), and curd firmness (A_{30})] were assessed by reversed-phase HPLC and computerized renneting meter analysis, respectively, using single test-day milk samples of 536 animals. Alternative protein variants of α_{S1} -CN and κ -CN were detected by HPLC, and identification of the corresponding genetic variants was carried out by DNA analysis. Two genetic variants were detected at *CSN1S1* (A and B variants) and 2 at *CSN3* (X1 and X2 variants). Statistical inference was based on a linear model including the *CSN1S1-CSN3* composite genotype effect (7 genotypes), the effects of herd-test-day (8 levels), and a combined days in milk (DIM)-parity class. Composite genotype AB-X2X2 was associated with decreased test-day milk yield [−0.21 standard deviation (SD) units of the trait] relative to genotype BB-X2X2. Genotypes did not affect milk protein content, but genotype AB-X1X1 was associated with increased fat content compared with genotype BB-X2X2 (+0.28 SD units of the trait) and AB-X1X1 (+0.43 SD units of the trait). For RCT, the largest difference (+1.91 min; i.e., 0.61 SD units of the trait) was observed between genotype AA-X1X2 and AB-X1X1. Direction of genotype effects on K_{20} was consistent with that for RCT. The maximum variation in K_{20} due to genotype effects (between AA-X1X2 and AB-X1X1 genotypes) was almost 0.9 SD units of the trait. Magnitude of genotype effects was smaller for A_{30} than for RCT and K_{20} , with a maximum difference of 0.5 SD units of the trait between genotype AA-X1X2 and AA-X1X1. The B allele at *CSN1S1* was associated with increased RCT and K_{20} and with weaker curds

compared with allele A. Allele X2 at *CSN3* exerted opposite effects on MCP relative to *CSN1S1* B. Because of linkage disequilibrium, allele B at *CSN1S1* and allele X2 at *CSN3* tend to be associated and this likely makes their effects cancel each other. This study indicates a role for casein genes in variation of MCP of buffalo milk. Further studies are necessary to estimate the effects of casein genetic variants on variation of cheese yield.

Key words: *CSN1S1*, *CSN3*, milk coagulation properties, water buffalo

INTRODUCTION

In the last 5 decades, the worldwide buffalo population has doubled and the Italian population of Mediterranean water buffalo has grown more than 10-fold (FAOSTAT, 1969–2009). In Italy, buffalo milk is mainly manufactured for the production of “Mozzarella di Bufala Campana,” a protected denomination of origin cheese and economically the third most important cheese product after Parmigiano Reggiano and Grana Padano (ISMEA, 2009).

Buffalo milk provides increased cheese yield relative to bovine milk and this feature is not exclusively attributable to the increased DM content of buffalo milk. A 1% increase in milk protein percentage is associated with an increase in cheese yield of more than 5% for buffalo milk and of almost 3% for bovine milk. This difference is smaller, but still detectable, when variation in fat content is accounted for (Zicarelli et al., 2001).

To date, few studies (Potena et al., 2007; Ariota et al., 2009) have investigated, using a limited number of samples, variation of traits correlated to cheese yield of buffalo milk, even though selective breeding of dairy buffalos relies on milk quality traits as indicators of cheese yield. Studies on bovine milk (Bonfatti et al., 2010a) detected relevant effects of milk protein genes and composition on milk coagulation properties (MCP), and gene-assisted breeding programs to exploit variation in milk protein genes have been suggested (Heck et al., 2009).

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¹Corresponding author: valentina.bonfatti@unipd.it

Genetic polymorphism in the κ -CN gene (*CSN3*) has never been reported for the Mediterranean water buffalo, but Feligini et al. (2009) recently detected a heteromorphism by using reversed-phase (RP)-HPLC techniques. Even though some protein genetic variants have been identified in the α_{S1} -CN gene (*CSN1S1*; Chianese et al., 2009), effects of these genetic polymorphisms on milk production traits (MPT) and MCP have never been investigated in buffalo. The aim of this study was to estimate the effects of *CSN1S1-CSN3* composite genotypes on MPT and MCP in Mediterranean water buffalo.

MATERIALS AND METHODS

Milk Sampling

Single test-day milk samples of 536 Mediterranean water buffaloes were collected from October 2010 to December 2010. Buffaloes were reared in 8 commercial herds in the Campania region of southern Italy. One composite milk sample from consecutive morning and evening milkings was collected for each animal. Each sample was partitioned into 2 subsamples, frozen immediately after collection, and stored at -20°C until the transfer to the laboratory for MCP and HPLC analysis. Samples were stored on dry ice during transfer and one subsample was thawed at 35°C for MCP analysis upon arrival. The remaining subsample was preserved at -40°C until RP-HPLC analysis. The Italian Water Buffalo Breeders Association (ANASB, Caserta, Italy) supplied milk recording data.

RP-HPLC Assessment of Genotypes at *CSN1S1* and *CSN3* Genes

Genotypes of buffaloes at *CSN1S1* and *CSN3* were assessed using the RP-HPLC method developed by Bonfatti et al. (2008) for bovine milk. Identification of each protein fraction was carried out by comparing the elution times of buffalo proteins with those of bovine or buffalo proteins obtained in previous studies (Bonfatti et al., 2008; Feligini et al., 2009). Alternative protein variants of α_{S1} -CN and κ -CN have been detected, and identification of the corresponding genetic variants was carried out by DNA analysis of samples with known RP-HPLC profiles.

Genomic DNA Isolation from Blood Samples

Eleven animals, previously phenotyped for the milk protein polymorphisms by RP-HPLC, were selected for blood sampling and DNA analysis to confirm the RP-HPLC results. Blood samples were collected in EDTA-containing tubes and stored at -20°C until

use. Genomic DNA was isolated from 100 μL of blood by using the DNeasy Blood and Tissue kit (Qiagen, Milan, Italy) following the manufacturer's instructions. Genomic DNA concentration and quality (260/280 and 260/230 nm absorbance ratios) were checked by using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and gel electrophoresis. A ratio of 1.7 to 1.9 was considered evidence of high DNA purity.

DNA Sequencing

A 333-bp fragment containing exon 17 and partial intron 17 of *CSN1S1* and a 350-bp fragment corresponding to exon 4 and a part of intron 4 of *CSN3* were PCR amplified using *CSN1S1_F* (5'-TGGATGCCTATC-CATCTGGT-3') and *CSN1S1_R* (5'-CACTGCTC-CACATGTTCTG-3') primers (Chessa et al., 2007), and *CSN3_F* (5'-ATCATTATGGCCATTC-CACCAAAG-3') and *CSN3_R* (5'-GCCCCATTTC-GCCTTCTCTGTAACAGA-3') primers, respectively (Patel et al., 2007; Gangaraj et al., 2008). The amplification reaction was performed in a 20- μL mixture containing 4 μL of DNA solution (at least 30 to 40 ng), 16.5 pmol of each primer, 1.5 U of GoTaq Flexi DNA polymerase (Promega, Madison, WI), 200 $\mu\text{mol/L}$ deoxyribonucleotide triphosphate mix, 1.5 mmol/L MgCl_2 , and 1 $\times$ reaction buffer (final concentrations). The following PCR conditions were used: an activation step at 95°C for 3 min, 35 cycles of 30 s at 95°C , 45 s at 60°C or 58.4°C (for *CSN1S1* and *CSN3*, respectively), 45 s at 72°C , and a final extension step of 5 min at 72°C . Amplicons were resolved on 1% agarose gel. When a single band of the expected size was obtained, the template was directly sequenced by using F and R primers and the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) following manufacturer's instructions, on an ABI Prism 3100 sequencer (Applied Biosystems). Unincorporated terminators were removed by isopropanol precipitation. Chromatograms obtained from the sequencing were checked by using FinchTV version 1.4.0 free DNA sequencing chromatogram trace viewer (Geospiza Inc., Seattle, WA) and compared by means of the software DNASTAR (DNASTAR Inc., Madison, WI).

Milk Coagulation Properties

Rennet coagulation time (RCT), curd firming time (K_{20}), and curd firmness (A_{30}) of individual milk samples were measured by using a computerized renneting meter (CRM-48, Polo Trade, Monselice, Italy) within 3 h after sample thawing. Details on the equipment and procedures used to assess MCP can be found in

Bonfatti et al. (2010a). Measurement of milk pH (pH-Burette 24, Crison, Barcelona, Spain) was also carried out.

Statistical Analysis

GenePop software (version 4.0; <http://kimura.unimontp2.fr/~rousset/Genepop.htm>) was used to test the hypothesis of linkage equilibrium between *CSN1S1* and *CSN3* and of Hardy-Weinberg equilibrium genotype frequencies at both genes.

Statistical inference was based on marginal posterior densities of solutions, obtained through Bayesian procedures and performing numerical integration using the Gibbs sampler, of a linear model including the *CSN1S1-CSN3* composite genotype effect (7 genotypes) as well as nongenetic sources of variation: the effects of herd-test-day (8 levels) and of a combined DIM-parity class. To create combined DIM-parity classes, parity was grouped into 3 classes: first parity, second to fourth parity, and more than 4 parities. For first-parity cows, there were 5 DIM classes (<210 d, 210 to 240 d, 241 to 270 d, 271 to 300 d, and >300 d); for cows of parity 2, 3, or 4, there were 7 DIM classes (<80 d, 81 to 140 d, 141 to 180 d, 181 to 210 d, 211 to 240 d, 241 to 270 d, and >270 d); for cows of parity >4, there were 5 DIM classes (<180 d, 180 to 210 d, 211 to 240 d, 241 to 270 d, and >270 d). Because of the low number of first-parity animals with data in early lactation, including in the model the effect of parity and DIM separately would have resulted in biased estimates of effects.

To avoid biased estimates of allelic effects due to linkage disequilibrium between genes, effects of genotypes at all investigated genes should be estimated simultaneously in the analysis (Bovenhuis et al., 1992), but allelic effects at each locus might be confounded even when they are included simultaneously in the model (Ojala et al., 1997). For these reasons, we estimated the effects of *CSN1S1-CSN3* composite genotypes.

Missing information about DIM and parity occurred for 15 buffaloes whose records were discarded when estimating genotype effects. Prior to statistical analysis, all traits were centered to a mean of 0 and scaled to a standard deviation (SD) of 1.

In all Bayesian analyses, bounded uniform distributions were used as prior densities for composite genotype and nongenetic effects. Bounds were set to ± 30 SD for each trait. A prior scaled inverted chi-squared distribution was assumed for the residual variance. The hyperparameters of the scaled inverted chi-squared distribution (ν and s^2) were equal to -2 and 0 , respectively, making the prior distribution flat (Wang et al., 1994). Statistical inference was based on the marginal posterior density of unknown parameters of concern.

These parameters were the solutions of composite genotype effects and differences between solutions of genotype effects providing estimates of effects of single-gene genotypes. Features of marginal posterior densities of concern were estimated using a single chain of 60,000 Gibbs samples with a burn in of 10,000 iterations. Convergence was checked by visual inspection of the chains. The posterior median was used as a point estimate of the parameter, and the highest 95% posterior density intervals were calculated. The posterior probability (P_0) of an estimate of being >0 (for positive estimates) or <0 (for negative estimates) and the posterior probability (P_R) of an estimate of being >0.1 (for positive estimates) or <-0.1 (for negative estimates) SD units of the trait were calculated.

To avoid biased estimates of genotype effects, the additive polygenic effect of animals or, alternatively, sire effects should be accounted for by the statistical model (Kennedy et al., 1992). Because AI is not a common practice in water buffalo, availability of reliable pedigree information is a very critical issue and misleading paternity identification can occur (Rosati and Van Vleck, 2002). In this study, sire-offspring relationships were ascertained through DNA testing for only about half of the animals. Hence, sire effects were not included in the linear model of analysis.

To avoid the possible confusion between the effect of genotype and herd effect, herds were selected on the basis of the presence of AI sires. A total of 17 AI sires (contributing 17% of the total number of samples) ensured connections among herds.

RESULTS AND DISCUSSION

Descriptive Statistics

Descriptive statistics for MPT and MCP are reported in Table 1. Average milk yield was lower than that reported for Mediterranean water buffalo by Zicarelli et al. (2001), whereas protein and fat contents were higher. Because most buffaloes were in mid or late lactation, lower yield and greater milk fat and protein contents relative to the findings of Zicarelli et al. (2001) were expected. Averages for MPT were in agreement with those reported by Polidori et al. (1997) for animals of similar lactation stage.

Average milk pH was in agreement with that reported by Ariota et al. (2007) for Mediterranean water buffalo. Almost 25% of samples exhibited very low milk pH (pH <6.50), only 2% of samples had pH >6.80 , and no sample showed pH ≥ 7 , which is considered a critical threshold for occurrence of milk rennet coagulation. Milk exhibited very favorable MCP. Most samples (83%) coagulated within 11 min of rennet addition,

Table 1. Descriptive statistics for yield, fat and protein contents, and coagulation properties of buffalo milk¹

Trait ²	Samples, no.	Mean	SD	P1	P99
Milk, kg/d	521	6.44	3.18	1.00	15.70
Protein, %	514	4.82	0.42	3.92	5.94
Fat, %	514	10.19	1.92	6.22	14.83
RCT, min	534	8.80	3.12	5.00	22.28
K ₂₀ , min	534	0.98	0.36	0.50	2.28
A ₃₀ , mm	534	41.79	5.29	29.00	54.00
pH	536	6.57	0.10	6.35	6.87

¹P1 = 1st percentile; P99 = 99th percentile.²RCT = rennet clotting time; K₂₀ = curd firming time; A₃₀ = curd firmness.

whereas less than 1% of the samples coagulated after 25 min (i.e., poorly coagulating milk). In the study of Ariota et al. (2007), average RCT and A₃₀ were greater than that measured in the current study. Few studies have investigated variation of buffalo milk MCP, and comparisons across studies are difficult because of the large variability due to the use of different measuring devices. All samples coagulated within 30 min of rennet addition, leading to normal distributions for RCT and A₃₀, in contrast to censored distributions obtained for the same traits in cattle due to the presence of noncoagulating milk (Ikonen et al., 2004). Shorter RCT and greater cheese yield of buffalo milk compared with bovine milk have been ascribed to high density and sensitivity to chymosin of buffalo casein micelles, and to increased casein content and degree of phosphorylation of α_{S2} -CN (Ferranti et al., 1996).

Herd, parity, and lactation stage accounted jointly for 54, 20, 30, 21, 13, 15, and 6% of the variation of milk yield, protein content, fat content, RCT, K₂₀, A₃₀, and pH, respectively.

Correlations for the Investigated Traits

Pearson product-moment correlations for the investigated traits are reported in Table 2. Consistent with Rosati and Van Vleck (2002), correlations of milk yield with protein and fat content were negative. Rennet clotting time was negatively correlated with milk yield and positively, although weakly, correlated with

protein and fat contents. Based on the estimated correlations, increased milk yield, protein content, and fat content would result in increased cheese yield because of increased RCT. Because rapidly coagulating milk tends to exhibit increased whey loss during curd formation (Potena et al., 2007), cheese yield of buffalo milk increases when RCT increases. Protein content was weakly and positively correlated with A₃₀, whereas fat content was uncorrelated to the strength of the curd.

In agreement with results of Potena et al. (2007), RCT was weakly correlated with A₃₀ ($r = -0.09$; $P < 0.05$). This is not consistent with results from studies on coagulation properties of bovine milk, for which marked and negative associations between RCT and A₃₀ were detected. The correlation between these traits was -0.87 in Finnish Ayrshire (Ikonen et al., 2004), -0.76 in Italian Holstein-Friesian (Cassandro et al., 2008), and -0.70 in Simmental cows (V. Bonfatti, unpublished data). Because the time frame for the observation of coagulation is restricted to 31 min, milk samples showing increased RCT have less time for curd firming and, thus, weaker curds than milk with short RCT. Results of studies on bovine milk indicate that the association between RCT and A₃₀ is tighter for breeds exhibiting increased RCT averages than for breeds having low RCT averages. Because of the low average RCT, the relationship between RCT and A₃₀ was expected to be weak for buffalo milk, with the firmness of the curd being independent of the variation of the time for the onset of coagulation.

Table 2. Pearson product-moment correlations for milk production traits and coagulation properties¹

	Protein	Fat	RCT	K ₂₀	A ₃₀	pH
Milk	-0.24***	-0.30***	-0.42***	-0.23***	-0.06	-0.08
Protein		0.38***	0.15***	0.07	0.12**	-0.03
Fat			0.14***	0.08	-0.01	0.03
RCT				0.47***	-0.09*	0.57***
K ₂₀					-0.37***	0.35***
A ₃₀						-0.12**

¹RCT = rennet clotting time; K₂₀ = curd firming time; A₃₀ = curd firmness.* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$: significance level of each correlation of being different from zero.

Table 3. Allele and genotype frequencies for *CSN1S1* and *CSN3* genes (n = 536)

Gene	Genotype frequency		Allele frequency	
	Genotype	Frequency, %	Allele	Frequency, %
<i>CSN1S1</i>	AA	11.6	A	34.3
	AB	45.5	B	65.7
	BB	42.9		
<i>CSN3</i>	X1X1	9.5	X1	31.5
	X1X2	44.0	X2	68.5
	X2X2	46.5		

Consistent with findings in bovine milk (Ikonen et al., 2004; Cassandro et al., 2008), a marked positive relationship between pH and RCT was estimated for buffalo milk, confirming the primary role of pH in the milk coagulation process. The correlation between pH and A_{30} was weak and slightly negative. For bovine milk, the correlation between pH and A_{30} was markedly negative (Ikonen et al., 2004; Cassandro et al., 2008; V. Bonfatti, unpublished data) as a consequence of the marked negative association between RCT and A_{30} .

Identification and Frequency of Polymorphisms at *CSN1S1* and *CSN3*

The RP-HPLC analysis of milk samples led to different protein patterns across individuals. Two alternative forms of α_{S1} -CN were detected. Genotyping results obtained through *CSN1S1* DNA sequencing perfectly matched with RP-HPLC results. Analysis of *CSN1S1* exon 17 showed the occurrence of a C > T transversion at nucleotide 578 of *Bubalus bubalis CSN1S1* complete coding sequence EMBL no. AJ005430.1. This polymorphism results in the AA substitution Leu¹⁷⁸(A) → Ser¹⁷⁸(B) of the mature polypeptide chain (from exon 3 to exon 17 of the reference sequence O62823). This substitution has been previously detected in Mediterranean water buffalo (Ferranti et al., 1998; Chianese et al., 2009).

Table 4. Observed and expected *CSN1S1-CSN3* composite genotype frequencies ($P < 0.001$)¹

Genotype	Frequency, %	
	Observed	Expected
AA-X1X1	7.46	1.10
AA-X1X2	3.92	5.09
AA-X2X2	0.19	5.37
AB-X1X1	1.87	4.33
AB-X1X2	36.38	20.05
AB-X2X2	7.28	21.15
BB-X1X1	0.19	4.08
BB-X1X2	3.73	18.89
BB-X2X2	38.99	19.94

¹Expected frequencies were calculated assuming linkage equilibrium between *CSN1S1* and *CSN3* genes.

Two alternative protein forms were detected in the elution interval of κ -CN. Sequencing of *CSN3* exon 4 demonstrated that all samples had a BB genotype for the presence of a C at nucleotide 506 of the complete coding sequence HQ677596. Hence, all samples had Ala at AA position 148 of the mature polypeptide chain (exons 3 and 4 of ADV40929 protein) instead of Asp (allele A). However, in agreement with Mitra et al. (1998), a T > C transversion at nucleotide 467 of the complete coding sequence HQ677596 was detected; T corresponds to allele X1, whereas C corresponds to allele X2. This results in the AA substitution Ile¹³⁵(X1) → Thr¹³⁵(X2) of the mature polypeptide chain. Results of RP-HPLC and DNA sequencing matched perfectly, and the 2 κ -CN peaks were attributed to the X1 and X2 genetic variants found by Mitra et al. (1998). Previous studies (Addeo et al., 1977; Chianese et al., 2009) reported that buffalo milk κ -CN is composed of 7 fractions showing variable degrees of phosphorylation or glycosylation, but genetic polymorphisms were never detected for this protein in the Mediterranean water buffalo. A heteromorphism was detected for κ -CN through RP-HPLC only by Feligini et al. (2009), but it was not confirmed by other studies. Because the substitution Thr¹³⁵→Ile¹³⁵ does not alter the net charge of the protein, such a polymorphism cannot be distinguished by electrophoretic techniques (Mitra et al., 1998) and no studies have investigated this polymorphism in the Mediterranean water buffalo by DNA sequencing. A neutral substitution (a C > T transversion) at the third base position of codon 136 was also observed, as previously reported by Mitra et al. (1998), and its presence was associated with the presence of allele X1.

For β -CN, α -LA, β -LG, and α_{S2} -CN, a unique RP-HPLC peak for each protein fraction was obtained. This result was expected for β -CN because 2 genetic variants (A and B) of β -CN have been observed so far, but variant A was identified only in a Venezuelan buffalo population (Ferranti et al., 1998). Conversely, 2 genetic variants were identified for α -LA in previous studies (Chianese et al., 2004; Patel et al., 2007). These protein variants were not detectable by RP-HPLC. This issue is of trivial importance because the minor

Table 5. Features of the marginal posterior density of *CSN1S1-CSN3* composite genotype effects relative to genotype BB-X2X2 on milk production traits (magnitude of effects is expressed in SD units of traits)¹

Genotype	Milk			Protein			Fat		
	Estimate	HPD95%	P _R	Estimate	HPD95%	P _R	Estimate	HPD95%	P _R
AA-X1X1	-0.04	-0.23, 0.16	30	0.02	-0.26, 0.30	30	-0.16	-0.41, 0.10	65
AA-X1X2	-0.10	-0.37, 0.16	50	0.01	-0.34, 0.38	34	0.12	-0.20, 0.47	56
AB-X1X1	-0.06	-0.43, 0.32	44	0.23	-0.30, 0.72	66	0.28	-0.22, 0.74	72
AB-X1X2	-0.04	-0.15, 0.08	19	-0.14	-0.30, 0.02	65	0.02	-0.12, 0.17	21
AB-X2X2	-0.21	-0.42, -0.01	83	0.02	-0.26, 0.32	33	0.00	-0.25, 0.28	27
BB-X1X2	-0.12	-0.37, 0.16	54	-0.12	-0.50, 0.29	53	0.09	-0.26, 0.47	48

¹Estimate = median of the marginal posterior density; HPD95% = highest 95% posterior density interval; P_R = posterior probability of the estimate of being >0.1 (for positive estimates) or <-0.1 (for negative estimates) SD units of the trait.

allele frequency (allele A) was reported to be 0.5% in Mediterranean water buffalo (Chianese et al., 2004). Based on this evidence, the *ALA* gene was considered virtually monomorphic. Even with the availability of reliable genotyping techniques, estimating genotype effects at *ALA* would have not been feasible because of the very limited number of homozygous *ALA-AA* samples. As for β -CN, unique peaks for β -LG and α_{S2} -CN were expected. Two β -LG variants have been to date in Indian and Egyptian populations (Otaviano et al., 2005; Patel et al., 2007; Karimi et al., 2009), but no polymorphism has been described for β -LG and α_{S2} -CN in the Mediterranean water buffalo (Ferranti et al., 1998; Feligini et al., 2009).

Allele and genotype frequencies for *CSN1S1* and *CSN3* are reported in Table 3. Allele frequencies were slightly unbalanced for both genes, with variants B and X2 being prevalent in *CSN1S1* and *CSN3*, respectively. Homozygous *CSN1S1* AA as well as *CSN3*

X1X1 individuals accounted for approximately 10% of the animals. Allele frequencies at *CSN1S1* were consistent with those reported by Chianese et al. (2004). The most frequent *CSN1S1-CSN3* composite genotypes were genotypes BB-X2X2 and AB-X2X2, comprising more than 75% of the sample. For *CSN3*, 2 different loci have been investigated so far: nucleotides 467 and 506 of the complete coding sequence HQ677596. The first locus was reported to be polymorphic in Indian river buffalo by Mitra et al. (1998) and Patel et al. (2007), but not by Gangaraj et al. (2008). The second one is consistently monomorphic (Mitra et al., 1998; Otaviano et al., 2005; Abdel Dayem et al., 2009).

Genotype frequencies at both genes were consistent with expected frequencies under Hardy-Weinberg equilibrium. Linkage disequilibrium between *CSN1S1* and *CSN3* was detected ($P < 0.001$). Differences between observed and expected frequencies of *CSN1S1-CSN3* composite genotypes (Table 4) indicated that *CSN1S1*

Table 6. Features of the marginal posterior density of the difference between *CSN1S1-CSN3* composite genotypes effects on milk production traits (magnitude of effects is expressed in SD units of traits)¹

Trait	Contrast ²	Estimate	HPD95%	P ₀	P _R
Milk	<i>CSN1S1</i> AB vs. AA	0.02	-0.22, 0.27	55	28
	<i>CSN1S1</i> BB vs. AB	0.07	-0.08, 0.25	76	39
	<i>CSN3</i> X1X2 vs. X1X1	-0.01	-0.25, 0.24	56	30
	<i>CSN3</i> X2X2 vs. X1X2	-0.01	-0.21, 0.13	62	27
Protein	<i>CSN1S1</i> AB vs. AA	0.05	-0.29, 0.36	58	37
	<i>CSN1S1</i> BB vs. AB	0.00	-0.24, 0.24	51	23
	<i>CSN3</i> X1X2 vs. X1X1	-0.19	-0.52, 0.14	81	66
	<i>CSN3</i> X2X2 vs. X1X2	0.14	-0.10, 0.36	83	62
Fat	<i>CSN1S1</i> AB vs. AA	0.18	-0.14, 0.47	82	65
	<i>CSN1S1</i> BB vs. AB	0.03	-0.19, 0.27	60	31
	<i>CSN3</i> X1X2 vs. X1X1	0.02	-0.30, 0.33	54	34
	<i>CSN3</i> X2X2 vs. X1X2	-0.06	-0.28, 0.16	67	36

¹Estimate = median of the marginal posterior density of the difference; HPD95% = highest 95% posterior density interval; P₀ = posterior probability of the estimate of being >0 (for positive estimates) or <0 (for negative estimates); P_R = posterior probability of the estimate of being >0.1 (for positive estimates) or <-0.1 (for negative estimates) SD units of the trait.

²*CSN1S1* AB vs. AA was computed as [(AB-AA + AB-AB)/2] - [(AA-AA + AA-AB)/2]; *CSN1S1* BB vs. AB was computed as [(BB-AB + BB-BB)/2] - [(AB-AB + AB-BB)/2]; *CSN3* X1X2 vs. X1X1 was computed as [(AA-X1X2 + AB-X1X2)/2] - [(AA-X1X1 + AB-X1X1)/2]; *CSN3* X2X2 vs. X1X2 was computed as [(AB-X2X2 + BB-X2X2)/2] - [(AB-X1X2 + BB-X1X2)/2].

Table 7. Features of the marginal posterior density¹ of *CSN1S1-CSN3* composite genotypes effects on milk coagulation properties² and pH (magnitude of effects is expressed in SD units of traits)

Genotype	RCT, min			K ₂₀ , min			A ₃₀ , mm			pH		
	Estimate	HPD95%	P _R	Estimate	HPD95%	P _R	Estimate	HPD95%	P _R	Estimate	HPD95%	P _R
AA-X1X1	0.26	-0.02, 0.54	83	0.58	0.29, 0.88	100	-0.15	-0.33, 0.01	71	0.03	-0.26, 0.32	35
AA-X1X2	-0.15	-0.52, 0.21	58	-0.26	-0.62, 0.12	75	0.34	0.12, 0.56	96	-0.25	-0.63, 0.14	74
AB-X1X1	0.47	-0.04, 0.99	88	0.63	0.13, 1.15	96	-0.00	-0.33, 0.31	31	0.67	0.13, 1.23	96
AB-X1X2	0.03	-0.13, 0.19	25	0.16	-0.01, 0.32	72	0.01	-0.09, 0.10	6	0.07	-0.09, 0.24	38
AB-X2X2	0.22	-0.07, 0.52	75	0.10	-0.19, 0.41	51	0.02	-0.16, 0.19	21	0.26	-0.04, 0.56	80
BB-X1X2	0.38	-0.02, 0.74	88	0.49	0.11, 0.88	96	-0.11	-0.33, 0.11	55	-0.02	-0.40, 0.39	37

¹Estimate = median of the marginal posterior density; HPD95% = highest 95% posterior density interval; P_R = posterior probability of the estimate of being >0.1 (for positive estimates) or <-0.1 (for negative estimates) SD units of the trait.

²RCT = rennet clotting time; K₂₀ = curd firming time; A₃₀ = curd firmness.

B was more frequently associated with *CSN3* X2 than with *CSN3* X1. Because AA-X2X2 and BB-X1X1 *CSN1S1-CSN3* composite genotypes were observed in just 1 animal each, records of those 2 animals were discarded from the analysis.

Effect of Casein Genotypes on Milk Production Traits

Features of the estimated marginal posterior density for the effects of *CSN1S1-CSN3* composite genotypes on MPT relative to BB-X2X2 genotype are reported in Table 5. Pedigree information was not available for these data, and sire-offspring relationships were known for approximately half of the animals. Consequently, it was not feasible to estimate *CSN1S1-CSN3* composite genotype effects while accounting for additive genetic effects of animals or, alternatively, sire effects. This might have resulted in biased estimates of genotype effects (Kennedy et al., 1992). However, due to the small size of sire families (from 1 to 4 daughters), bias was expected to be small.

Relative to that of genotype BB-X2X2, decreased test-day milk yield (-0.21 SD) was observed for genotype AB-X2X2. The posterior probability for this effect being relevant (i.e., <-0.1 SD) was 83%. Genotypes did not affect milk protein content, but the probability of relevance for genotype effects was not small for fat content. In particular, milk fat content of AB-X1X1 animals was 0.83 percentage points greater than fat content of AA-X1X1 animals.

When differences among single-gene genotypes were estimated to better evaluate the role of each gene in affecting variation of milk production traits, no relevant effects were observed (Table 6). These results indicate that influences of single-gene additive effects are not relevant for milk production traits, but across-genotype variations of these traits and of fat content are attributable to specific gene-by-gene interactions.

Effect of Casein Genotypes on Milk Coagulation Properties and pH

Features of the estimated marginal posterior density of composite genotype effects on MCP and pH relative to genotype BB-X2X2 are reported in Table 7. Milk coagulation properties differed across genotypes. Animals of genotype AA-X1X2 exhibited the shortest RCT. Genotypes AB-X1X1, BB-X1X2, and AA-X1X1 had longer RCT (+1.46, +1.18, and +0.82 min, respectively) relative to genotype BB-X2X2. The maximum RCT difference across genotypes was estimated to be 1.91 min (i.e., >0.6 SD) and was observed between genotype

Table 8. Features of the marginal posterior density of the difference between composite *CSN1S1-CSN3* genotypes effects on milk coagulation properties and pH (magnitude of effects is expressed in SD units of traits)¹

Trait ²	Contrast ³	Estimate	HPD95%	P ₀	P _R
RCT	<i>CSN1S1</i> AB vs. AA	0.20	-0.14, 0.53	83	68
	<i>CSN1S1</i> BB vs. AB	0.06	-0.19, 0.30	66	39
	<i>CSN3</i> X1X2 vs. X1X1	-0.42	-0.77, -0.07	98	94
	<i>CSN3</i> X2X2 vs. X1X2	-0.10	-0.33, 0.16	74	49
K ₂₀	<i>CSN1S1</i> AB vs. AA	0.22	-0.11, 0.56	86	74
	<i>CSN1S1</i> BB vs. AB	0.11	-0.14, 0.36	79	55
	<i>CSN3</i> X1X2 vs. X1X1	-0.64	-1.00, -0.31	100	100
	<i>CSN3</i> X2X2 vs. X1X2	-0.28	-0.53, -0.03	97	88
A ₃₀	<i>CSN1S1</i> AB vs. AA	-0.10	-0.28, 0.10	79	49
	<i>CSN1S1</i> BB vs. AB	-0.07	-0.20, 0.07	79	35
	<i>CSN3</i> X1X2 vs. X1X1	0.26	0.05, 0.45	99	90
	<i>CSN3</i> X2X2 vs. X1X2	0.06	-0.08, 0.20	76	33
pH	<i>CSN1S1</i> AB vs. AA	0.47	0.14, 0.85	99	97
	<i>CSN1S1</i> BB vs. AB	-0.17	-0.40, 0.09	86	69
	<i>CSN3</i> X1X2 vs. X1X1	-0.44	-0.80, -0.06	97	93
	<i>CSN3</i> X2X2 vs. X1X2	0.10	-0.15, 0.34	73	51

¹Estimate = median of the marginal posterior density; HPD95% = highest 95% posterior density interval; P₀ = posterior probability of the estimate of being >0 (for positive estimates) or <0 (for negative estimates); P_R = posterior probability of the estimate of being >0.1 (for positive estimates) or <-0.1 (for negative estimates) SD units of the trait.

²RCT = rennet clotting time; K₂₀ = curd firming time; A₃₀ = curd firmness.

³*CSN1S1* AB vs. AA was computed as [(AB-AA + AB-AB)/2] - [(AA-AA + AA-AB)/2]; *CSN1S1* BB vs. AB was computed as [(BB-AB + BB-BB)/2] - [(AB-AB + AB-BB)/2]; *CSN3* X1X2 vs. X1X1 was computed as [(AA-X1X2 + AB-X1X2)/2] - [(AA-X1X1 + AB-X1X1)/2]; *CSN3* X2X2 vs. X1X2 was computed as [(AB-X2X2 + BB-X2X2)/2] - [(AB-X1X2 + BB-X1X2)/2].

AA-X1X2 and AB-X1X1. Genotypes effects on K₂₀ exhibited the same direction of those estimated for RCT. The maximum change in K₂₀ due to genotype effects was 0.9 SD units of the trait and was detected when comparing genotype AA-X1X2 and AB-X1X1. In addition to shortest RCT and K₂₀, genotype AA-X1X2 also exhibited the largest A₃₀. This was the only relevant genotype effect affecting variation of A₃₀. For A₃₀, the magnitude of genotype effects was smaller than that for RCT and K₂₀, with a maximum difference across genotypes of 0.5 SD units of the trait.

Although *CSN1S1-CSN3* genotypes affect variability of milk pH, effects of genotypes on MCP cannot be ascribed to changes in milk acidity, the only exception being the high RCT for genotype AB-X1X1, which might be attributed in part to the increased pH observed for this genotype.

When comparisons between single-gene genotypes were performed to evaluate the role of each gene in determining variation of MCP, relevant differences were detected (Table 8). Genotypes carrying the B allele at *CSN1S1* were associated with longer RCT and K₂₀ and weaker curds compared with genotypes carrying variant A. Conversely, *CSN3* X2 was associated with shorter RCT and K₂₀ and with increased A₃₀ relative to *CSN3* X1. Because *CSN1S1* B and *CSN3* X2 tend to be associated due to linkage disequilibrium, their effects partially cancel each other. Likely, effects of these

genes would have been more apparent in the absence of linkage.

In dairy cattle, the genetic variant B of κ -CN is associated with lower values of RCT and increased A₃₀ compared with variant A (Bonfatti et al., 2010b) as a consequence of the increased proportion of κ -CN in CN associated with allele B (Bonfatti et al., 2010b, 2011). Based on this evidence, we hypothesized that, in buffalo, the effects of CN genes on MCP are likely due to the variation in milk protein composition associated with specific CN genetic variants.

CONCLUSIONS

We confirmed the existence of *CSN1S1* and *CSN3* genetic variants in Mediterranean water buffalo and investigated their associations with variation of MPT and MCP of milk. The linkage detected between *CSN1S1* and *CSN3* did not allow us to obtain unbiased estimates of single gene effects. However, this study indicated a possible role of CN genes in variation of MCP and cheese yield of buffalo milk. Further studies are necessary to investigate the role of milk protein genetic variants on variation in cheese yield. Moreover, the effect exerted by *CSN1S1-CSN3* composite genotypes on variation of protein composition of buffalo milk would be of interest to elucidate the role of *CSN1S1* and *CSN3* in cheese-making ability of buffalo milk.

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