Mediterranean River Buffalo *CSN1S1* gene: search for polymorphisms and association studies

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Abstract. The aim of the present work was to study the variability at *CSN1S1 locus* of the Italian Mediterranean river buffalo and to investigate possible allele effects on milk yield and its composition. Effects of parity, calving season and month of production were also evaluated. Three SNPs were detected. The first mutation, located at position 89 of 17th exon (c.628C>T), is responsible for the amino acid change p.Ser178 (B allele)/Leu178 (A allele). The other two polymorphisms, detected at the positions 144 (c.882G>A) and 239 (c.977A>G) of 19th exon respectively, are silent (3' UTR).

Associations between the *CSN1S1* genotypes and milk production traits were investigated using 4,122 test day records of 503 lactations from 175 buffalo cows. Milk yield, fat and protein percentages

27 were analyzed using a mixed linear model. A significant association between the c.628C>T SNP and

28 the protein percentage was found. In particular, the CC genotype showed an average value of about

29 0.04% higher than the CT and TT genotypes. The allele substitution effect of the cytosine into the

30 thymine was -0.014, with a quite low (0.3%) protein percentage (PP) contribution on total phenotypic

31 variance. A large dominance effect was detected.

32 Furthermore, a characterization of the *CSN1S1* transcripts and a method based on *Mbo*I-ACRS-

33 PCR for a rapid genotyping of c.628C>T were provided.

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35 **Additional keywords**: Mediterranean river buffalo, *Bubalus bubalis*, *CSN1S1*, milk protein

36 percentage, marker assisted selection

37

Introduction

39 Historically domestic water buffalo were divided into swamp and river subspecies that differ in

40 morphology, behaviour, and chromosome number (2n=48 and 2n=50, respectively) (Ajmone-Masan P.

41 *et al*, 2013). In particular, the Mediterranean river buffalo, is the only indigenous Italian breed of water

42 buffalo. It is of the River subtype of water buffalo, and is genetically and ecological similar to the

43 buffalo breeds of Hungary, Romania and the Balkan countries. Previously considered to belong to the

44 Mediterranean buffalo group, it was officially recognised as a breed in 2000, based on its long isolation

45 period and lack of interbreeding with other buffalo breeds.

46 The improvement of animal performances represents a priority for the Italian dairy Buffalo

47 industry in order to fulfill the increasing market demand for mozzarella cheese. Italian buffalo stock

48 consists of approximately 344,000 Mediterranean river buffaloes (http://faostat.fao.org/). In 2011, the

49 average milk yield per lactation per buffalo cow (35,963 registered cows in the national herd book) was

50 kg 2,223 with 8.49 and 4.65 % of fat and protein content, respectively (http://www.aia.it/aia-

website/it/home). The milk is almost completely processed into cheese. A breeding program aimed at improving buffalo milk yield and composition is currently operating in Italy, however the low efficiency of the artificial insemination, the difficulties to detect the oestrus and the variability of its length are among the main causes of a very limited impact on the population (Barile, 2005).

Recent advances of molecular genetics offer the possibility to investigate genomic regions that affect traits of economic importance and to identify genetic polymorphisms useful for marker-assisted selection (MAS) programs. In the last decades, several association studies between milk production traits and markers located in milk protein genes have been carried out in cattle, sheep and goat (Ibeagha-Awemu *et al*, 2008; Martin *et al*, 2002; Mroczkowski *et al*, 2004). In particular, the goat αs1 encoding gene (*CSN1S1*) was found to be highly polymorphic with at least 17 alleles associated with qualitative and quantitative differences for the content of αs1casein (Ramunno *et al*, 2004; Ramunno *et al*, 2005), fat (Grosclaude *et al*, 1994; Chilliard *et al*, 2006), urea level (Schmidely *et al*, 2002; Bonanno *et al*, 2007; Avondo *et al*, 2009), fatty acid profile Chilliard *et al*, 2006) and milk yield (Yue *et al*, 2011). Associations between alleles at *CSN1S1 locus* and protein content were also observed for other species as for instance in bovine (Rando *et al*, 1998; Prinzenberg *et al*, 2003; Çardak, 2005) and ovine (Pirisi *et al*, 1999; Wessels *et al*, 2004).

In ruminant species, the *CSN1S1* gene is characterized by an extremely split architecture with 19 exons, many of which (exons 5, 6, 7, 8, 10, 13 and 16) of small size (24 bp) (Ramunno *et al*, 2004; Koczan *et al*, 1991; Calvo *et al*, 2011). In buffalo, the αs1-casein gene codes for a precursor of 214 amino acids with a signal peptide of 15 amino acid residues (Ferranti *et al*, 1998; Sukla *et al*, 2007). Currently, several partial or complete bubaline *CSN1S1* cDNA sequences are available in EMBL (FJ392261; AJ005430; AY948385; EF025981; EF025982; EF025983; DQ111783). The similarity between buffalo and cattle, goat and sheep αs1-casein mRNA sequence is 97.2, 93 and 92.3%,

74   respectively. A similar trend was observed comparing amino acid sequences of these species (Sukla *et*

75   *al*, 2007).

76   Few polymorphisms have been reported for the *CSN1S1 locus* in buffalo. The occurrence of a

77   αs1-casein B genetic variant characterized by a single amino acid substitution (p.Leu178Ser) as

78   consequence of single nucleotide substitutions was found for the first time in Romanian Buffalo breed

79   (Balteanu *et al*, 2008) and confirmed at amino acid level in Mediterranean water buffalo (Chianese *et*

80   *al*, 2009). Furthermore in Indian water buffalo a novel *CNS1S1* allele has been characterized by

81   c.620G>A substitution. It led to a p.Gly192Glu replacement in the peptide chain (Sukla *et al*, 2007).

82   Recently, additional SNPs were detected: a transition c.136G>A at exon 5, leading to a

83   p.Val31Met substitution, a transition c.175A>G at exon 7, leading to a p.Ile44Val substitution in the

84   peptide chain, one SNP (g.218T>C) in intron 5 and three SNPs (g.472G>C; g.547C>T; g.856T>C) in

85   intron 6. In particular, it was reported that the g.472G>C substitution inactivates the intron 6 splice

86   donor site promoting the skipping of exon 6 of the buffalo *CSN1S1* mRNA and, as consequence,

87   triggering the synthesis of a defective protein lacking eight amino acids (Balteanu *et al*, 2013).

88   In recent years, many studies have been carried out for the identification of the genetic

89   polymorphisms at the *loci* coding for the buffalo milk proteins (Cosenza *et al*, 2009a, b; Masina *et al,*

90   2007), and candidate genes responsible for the variation of the quali-quantitative characteristics of the

91   Mediterranean water buffalo milk have been found (Cosenza *et al,* 2007; Pauciullo *et al,* 2010). Efforts

92   in this direction also allowed to find significant associations with traits of economic interest, as milk

93   yield (Pauciullo *et al*, 2012a, b) and milk coagulation properties (Bonfatti *et al*, 2012b).

94   The aim of our work was to study the variability at the Italian Mediterranean river buffalo

95   *CSN1S1* cDNA and to investigate possible associations with milk yield and composition.

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97

98   **Materials and methods**

99   *Sampling*

100 Individual blood samples were collected from 175 Italian river buffaloes randomly chosen and

101 belonging to an experimental herd, located in Salerno province (Southern Italy).

102 In order to characterize the *CSN1S1* transcripts and to detected polymorphisms at this *locus*,

103 individual milk samples were collected from 10 animals at comparable age, in third calving, at 120

104 days in milking and free of clinical mastitis and randomly chosen in different farms located in the

105 province of Salerno and Caserta (Southern Italy). After collection, milk samples were immediately

106 frozen and kept at -80 °C until analysis. Sampling was carried out in collaboration with the Italian

107 National Association of Buffalo Breeds (ANASB).

108

109 *RNA extraction*

110 Total RNA was isolated from somatic cells (SCC range from 10,000 to 12,000/mL) present in

111 individual milk samples by using NucleoSpin® Extract Kits (Macherey-Nagel). A digestion with 2U of

112 DNase I (Ambion) in 1X DNase buffer was carried out according to the manufacture guidelines at

113 37°C for 30 min followed by the enzyme inactivation at 75°C for 5 min. The quantity, quality, purity,

114 and integrity of RNA after DNase treatment were estimated by means of Nanodrop 2000c

115 spectrophotometer (Thermo Scientific, Barrington, IL) and by electrophoresis on a denaturing agarose

116 gel.

117

118 *Reverse transcription, PCR and cloning*

119 Total RNA was converted into cDNA by reverse transcription using Improm- II Reverse Transcriptase

120 (Promega) with a final volume of 20µl. The reaction was performed using cDNA19R (5'-

5

121 CAAAATCTGTTACTGCACA - 3'), a reverse primer complementary to nt 327–345 of 19th exon of

122 buffalo *CSN1S1* cDNA sequence (accession number AY948385).

123   The PCR was performed using: cDNA19R and cDNA1F (5'- AACCCAGCTTGCTGCTT - 3'),

124 a forward primer corresponding to nt 1–17 of partial 1st exon of buffalo *CSN1S1* cDNA sequence

125 (accession number AY948385). The PCR reaction mix comprised 20 µl of RT reaction product, 50 mM

126 KCl, 10 mM Tris–HCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 10 pmol of each primer, dNTPs each at 0.2

127 mM, 5 U of Taq DNA Polymerase (Promega, Madison, WI), with a final volume of 100 µl.

128   The amplification protocol consisted of 39 cycles: the first cycle involved a denaturation step at

129 97 °C for 2 min, an annealing step at 57 °C for 30 s and an extension step at 72 °C for 1 min and 30 s.

130 The next 37 cycles were performed under the following conditions: 94 °C for 30 s, 57 °C for 30 s. and

131 72 °C for 1 min and 30 s. In the 39th cycle, the final extension step was carried out at 72 °C for 10 min.

132   The amplified products were first analyzed by electrophoresis on 3% agarose gel in TBE 1X

133 buffer (Bio-Rad) and then cloned in pCR2.1-TOPO plasmid by using the TOPO TA cloning kit

134 (Invitrogen, Pro, Milan, Italy). White recombinant clones were randomly chosen and screened by PCR

135 using standard vector primers M13. Recombinant clones underwent plasmid purification by

136 PureYield[TM] Plasmid Midiprep System (Promega, USA) and then sequenced on both strands at

137 CEINGE - Biotecnologie Avanzate (Naples, Italy).

138

139 *DNA extraction*

140 DNA was extracted from leukocyte, using the procedure described by Gossens and Kan (1981).

141 Briefly, fresh buffy coat samples were washed twice with distilled water and NaCl 1.8% to remove the

142 excess of red cells, protein digestion was carried out with 500 µl of proteinase K solution (2 mg/ml of

143 proteinase K, 1% w/v SDS and 0.02 M EDTA). Proteins were extracted using phenol-chloroform

144 method followed by DNA precipitation with cold isopropanol. The isolated DNA was then resuspended

6

145   in 100 µl TE buffer pH 7.6 (10 mM Tris, 1mM EDTA). DNA concentration and $OD_{260/280}$ ratio of the

146   samples were then measured by the Nanodrop ND-2000C Spectrophotometer (Thermo Scientific).

147

148   *CSN1S1 locus genotyping*

149   In order to genotype 175 individual samples of water buffalo for the c628C>T mutation, a method

150   based on ACRS-PCR (Amplification Created Restriction Site PCR) was developed according to Lien *et*

151   *al* (1992). The ACRS-PCR was performed using: ACRS17F (5'- CAATACCCTGATGCCC*G*AT - 3')

152   as forward and ACRS17R (5'- CACCACAGTGGCATAGTAG - 3') as reverse, corresponding to nt

153   70–88 and complementary to nt 137–155 of the 17th exon of buffalo *CSN1S1* cDNA sequence (EMBL

154   HE573919), respectively. According to the method, the forward primer was modified by changing

155   C→G in position 17 in order to provide a restriction site for the *Mbo*I (!*G*ATC) endonuclease.

156   The amplification protocol consisted of 39 cycles: the first cycle involved a denaturation step at

157   95 °C for 5 min. The next 38 cycles were performed under the following conditions: 95 °C for 45 s,

158   63.2 °C for 45 s. and 72 °C for 20 s. In the 39th cycle, the extension step was carried out at 72 °C for 10

159   min. PCR reaction mix comprised: 100 ng of genomic DNA, 50mM KCl, 10mM Tris–HCl, 0.1%

160   Triton X-100, 3mM $MgCl_2$, 5 pmol of each primer, dNTPs each at 400 µM, 2.5U of *Taq* DNA

161   Polymerase (Promega, Madison, WI), and 0.04% BSA, with a final volume of 25 µl.

162

163   *Digestion and electrophoresis condition*

164   Each PCR amplification product was digested with 10U of *Mbo*I after incubation for 5 h at 37 °C;

165   following the supplier's guidelines. The restricted fragments were analyzed directly by electrophoresis

166   on 3% TBE agarose gel in 1 X TBE buffer and stained with ethidium bromide.

167

168   *Association study*

169   Associations between *CSN1S1* genotypes and milk production traits were carried out on 4,122 test day

170   records of 503 lactations from 175 buffalo cows, supplied by the Italian Association of Buffalo

171   Breeders (ANASB). Milk yield (MY), fat (FP) and protein percentages (PP) were tested with the

172   following mixed linear model:

173

174    $$y_{ijklmno} = Month_i + Par_j + Sea_K + DIM_l + \alpha_{s1m} + c_n(\alpha_{s1m}) + e_{ijklmno} \qquad [*]$$

175

176   where: y_{ijkmno} is the test-day record of MY, FP or PP; *Month$_i$* is the fixed effect of the i-th month of

177   production (12 levels); *Par$_j$* is the fixed effect of the j-th parity (6 levels: 1 to 5, >5); *Sea$_K$* is the fixed

178   effect of the k-th calving season (4 levels: autumn, winter, spring, summer); *DIM$_l$* (Days In Milk) is the

179   fixed effect of the l-th stage of lactation (30 levels of 10 days each); α_{s1m} is the fixed effect of the m-th

180   genotype at the c.628C>T SNP of *CSN1S1* gene (3 levels: CC, CT, TT); c_n is the random effect of

181   individual cow (175 levels), nested within α_{s1} genotype; and $e_{ijklmno}$ is the random residual. Pairwise

182   comparisons among different levels of fixed effects included in model were performed using a

183   Bonferroni adjusted test. (Co)variance matrices of random effects of cow and residual were assumed to

184   be diagonal, $\mathbf{I}\sigma^2_c$ and $\mathbf{I}\sigma^2_e$, respectively. They allow for the REML estimation of variance components

185   associated to individual cow (σ^2_c) and residual (σ^2_e). Variance component associated to the α_{s1} *locus*

186   ($\sigma^2_{\alpha s1}$) was estimated running a mixed model having the same structure of [*] but with the α_{s1}

187   genotype treated as random. Contributions of α_{s1} *locus* ($r^2_{\alpha s1}$) and cow (r^2_c) to the total phenotypic

188   variance of the trait was calculated as the ratio between $\sigma^2_{\alpha s1}$ and σ^2_c, respectively and the sum of all

189   variance components (i.e. $\sigma^2_{\alpha s1} + \sigma^2_c + \sigma^2_e$).

190   In order to estimate the average of gene substitution effect (α) and a possible dominance effects

191   (d), gene effect was treated as a covariable, represented by the number of T alleles at the α_{s1} *locus* (0,

192 1, 2). Finally, an interaction between alleles at the SNP *locus* was considered (Banos *et al*, 2008;

193 Barendse *et al*, 2008).

194

195 **Results and discussion**

196

197   *a) Characterization of CSN1S1 transcripts*

198 *Analysis of the cloned RT-PCR fragments*

199 The mRNAs extracted from individual milk samples obtained from 10 Mediterranean river buffaloes,

200 randomly chosen in the province of Salerno and Caserta, was investigated through the clone analysis.

201 10 positive clones for each individual were screened. The electrophoretic analysis of the PCR products

202 and the subsequent sequencing of clones, showed at least two populations of transcripts for each

203 examined individual. The most represented population (about 90 %) was correctly assembled, followed

204 by that one deleted of the first triplet of the 11th exon. The last event is a constitutive allele independent

205 event which takes place during the maturation of the pre-mRNA. The first codon of exon 11 (CAG),

206 coding for glutamine in position 78, is in fact competitively eliminated from the mRNA because it is

207 recognized as cryptic site of splice instead of the canonic site (AG) located at the end of the 10th intron.

208 This feature was already observed in buffalo (Ferranti *et al*, 1999) and it is common event for the

209 *CSN1S1* gene of other ruminants, like sheep (Ferranti *et al*, 1998), goat (Ramunno *et al*, 2005) and

210 cattle (Ferranti *et al*, 1999). Furthermore, as already observed in the afore mentioned species, also for

211 the river buffalo it is reasonable to hypothesize the existence of other transcripts different from those

212 reported in this study. Their undetectable amounts opens a new opportunity of investigation in the field

213 of the transcript analysis for buffalo milk protein.

214

215 *Polymorphism detection*

In order to detect polymorphisms at the Mediterranean river buffalo *CSN1S1 locus*, the correctly assembled transcripts were sequenced. The analysis of the sequences showed a transcript of 1083 bp, spanning from the 16th nt of the 1st exon to the 345th nt of the 19th exon. The comparison of the obtained sequences (EMBL HE573919 and HE573920) showed 3 transitions. The first was located at the position 89 of the 17th exon (c.628C>T) and the other two at the position 144 and 239 of the 19th exon, respectively (c.882G>A and c.977A>G). The last two mutations are silent because they are located in the 3 'UTR, whereas the first is a miss-sense SNP (p.Ser178Leu). This amino acid change at the buffalo *CSN1S1 locus* was already observed and it characterizes the genetic variants of the αs1-casein, named B and A, respectively (Balteanu *et al*, 2008; Chianese *et al*, 2009).

Since the presence of the cytosine in position 628 characterizes even other *CSN1S1* sequences of buffalo (FJ392261, AY948385, AJ005430, DQ111783) and of other ruminants, such as goat (AJ504710.2), sheep (NM_001009795), bison (EU862388) and cattle (X59856), its presence might be indicative of an ancestral condition. According to the sequence analysis of the cDNA samples belonging to individuals with informative genotypes, the G in position 882 and the A in position 977 are in *cis* with the T at 89th nucleotide of exon 17. Although the complete genomic sequence of the river buffalo *CSN1S1* is not available, a close distance exists between the exons 17 and 19 in the homologous sequences of the other ruminants. For instance, this DNA region is only 1962 bp long in the bovine *CSN1S1* gene (EMBL acc. No. X59856), therefore, a condition of linkage disequilibrium can be assumed for these SNP in river buffalo.

b) Association of CSN1S1 polymorphism on milk yield and composition

Genotyping of Mediterranean river buffalo CSN1S1 alleles

The c.628C>T does not alter or create any restriction site, therefore, we established a screening method based on the *Mbo*I-ACRS-PCR to identify the carriers of this mutation in an easy and rapid way.

The amplified fragment includes the last 86 bp of 17th exon. Therefore, the digestion with such endonuclease produces an undigested fragment of 86 bp in individuals homozygous for the thymine and two fragments of 70 and 16 bp (not visible electrophoretically) in buffaloes homozygous for the cytosine. The heterozygous individuals produce a pattern characterized by all 3 restriction fragments: 86, 70 and 16 bp (Figure 1). The investigated population was in Hardy–Weinberg equilibrium. Genotype distribution and allele frequencies are reported in Table 1. The frequency of the thymine was 0.33. This result is in agreement with data reported by Chianese *et al* (2009) and Bonfatti *et al* (2012a) and it confirms that the most common genetic variant in Mediterranean breed reared in Campania (Italy) is the αs1-CN B variant.

Association study

All environmental factors included in model [*] affected significantly all the traits considered (Table 2) except the calving season for FP. The genotype at the *CSN1S1 locus* was significantly associated with protein percentage (p<0.04). In particular (Table 3), the CC genotype showed an average value of about 0.04% higher than the CT and TT genotypes. The allele substitution effect of a cytosine in a thymine was -0.014±0.014, with a quite low contribution of the *CSNSI locus* to the total phenotypic variance of PP ($r^2_{\alpha s1}$=0.003) PP. A large effect of dominance (-0.028±0.019) was also observed. Often such effect is not detected or considered non relevant because numerically much lower than the additive effect. Although dominance effects are not important in the estimation of breeding values, being not transmitted in the offspring, they might have an impact on allele substitution effect in the population as recently reported (Pauciullo *et al*, 2012a, b).

Recently, Bonfanti *et al* (2012a) estimated the effects of the *CSN1S1* (B and A alleles, c.628C>T transition) -CSN3 (k-casein, X1 and X2 alleles) genotypes on milk production traits and milk coagulation properties in Mediterranean water buffalo. In particular, these authors report that genotypes

264   did not affect milk protein content, but the composite genotype AB-X1X1, compared to genotype AA-

265   X1X1, was associated with an increased fat content and they indicate a role for the casein genes in the

266   variation of the coagulation properties of the buffalo milk. However, the same authors (Bonfatti *et al*,

267   2012b) reported that the increased proportion of the αs1-CN on the total casein (TCN) content is

268   associated with genotypes carrying the allele *CSN1S1* A. On the contrary, genotypes associated with a

269   marked decrease of the αs1-CN on the TCN (composite genotypes AB-X1X1 and BB-X1X2) are

270   associated with marked increases in the proportion of αs2-CN.

271   Although results of the present work need to be confirmed with large-scale studies, they might

272   be of great economic interest for the buffalo dairy industry. In fact, increases in average protein content

273   would lead consequently to an expected increase of mozzarella production.

274

Conclusions

276   The present study reports a characterization of the Mediterranean water buffalo *CSN1S1* transcripts. At

277   least two populations of transcripts were detected. The most represented population (about 90 %) was

278   correctly assembled, followed by that one deleted of the first triplet of the 11th exon.

279   Also the study confirms the existence of genetic polymorphisms at this *locus* and it offers a

280   method based on the *Mbo*I-ACRS-PCR for a rapid genotyping at DNA level for the *CSN1S1* A and B

281   alleles. Furthermore, a significant association between the c.628C>T SNP and the protein percentage

282   was found. In particular, the CC genotype showed an average value of about 0.04% higher than the CT

283   and TT genotypes.

284   Therefore, further studies are necessary to better determine the real effects of the transition

285   c.628C>T on milk composition. Besides, an investigation on larger population are needed in order to

286   validate its application.

287   In addition, it is necessary to investigate the remaining polymorphisms detected in the 3' un-

288 translated regions (UTR). In fact, it is well known that the sequences in the 3' UTR can affect the

289 mechanism of mRNA regulation, such as de-adenylation and degradation. Therefore, it is reasonable to

290 hypothesize that these observed mutations might influence, directly or indirectly, the gene expression

291 and, consequently, the milk protein composition.

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428   **Table 1.** Genotyping data and allele frequency of the c.628C>T SNP at the *CSN1S1* gene in the Italian

429   Mediterranean river buffalo population.

	Genotypes				Allelic frequency	
	CC	**CT**	**TT**	**Total**	**C**	**T**
Observed	75	84	16	**175**	0.67	0.33
Expected	78.22	77.55	19.22			

430   χ^2=1.20- p≤0.05 - d.o.f.=1

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433   **Table 2.** Statistical significance of factors included in model

	P-value		
Effect	Milk yield	Fat	Protein
Genotype	0.63	0.93	0.04
Parity	<.001	<.001	<.001
Month of production	<.001	<.001	<.001
DIM	<.001	0.02	<.001
Season	<.001	0.48	0.02

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436   **Table 3.** Least squares means of milk yield (kg/d), fat and protein percentage (%) for the genotypes at

437   the *locus* c.628C>T of river buffalo *CSN1S1* gene estimated with model [*].

Genotype	Animals n.	Milk yield (kg/d)	Fat (%)	Protein (%)
CC	75	7.81±0.17	9.22±0.12	4.72±0.016[a]
CT	84	7.92±0.17	9.27±0.12	4.68±0.016[b]
TT	16	7.56±0.36	9.20±0.23	4.69±0.029[ab]

438   [a,b] Means within columns with different superscripts differ (P=0.038)

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448 **Table 4.** Substitution effect of a cytosine with a thymine at the c.628C>T SNP in the *CSN1S1* gene

449 (mean ± SE) and contribution of the αs1 polymorphism to the phenotypic variance for protein

450 percentage.

Statistic	Value
α	-0.014±0.014
d	-0.028±0.019
$\sigma^2_{\alpha s1}$	0.0004
σ^2_{c}	0.0034
σ^2_{e}	0.1596
$r^2_{\alpha s1}$	0.003
r^2_{c}	0.021

451 α: Substitution effect;
452 d: dominance effect;
453 σ^2: variance components associated to the genotype (αs1); to the individual buffalo cow (c), to
454 residuals (e);
455 r^2: contributions of genotype (αs1) and of individual buffalo cow (c) to the total phenotypic variance.
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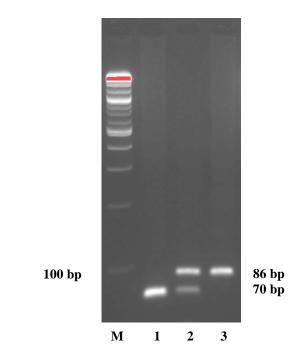461
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474 **Figure 1.** Observed genotypes after *Mbo*I digestion of fragments obtained by ACRS-PCR of a DNA
475 region corresponding to the last 86 bp of the 17th exon into Mediterranean river buffalo *CSN1S1* gene.
476 M=2-Log DNA ladder (0.1-10.0kb) (New England Biolabs); lane 1: *CSN1S1* C/C; lane 2: *CSN1S1*
477 C/T; lane 3 *CSN1S1* T/T