Italian Journal of Animal Science

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tjas20

A novel duplex ACRS-PCR for composite *CSN1S1–CSN3* genotype discrimination in domestic buffalo

Alfredo Pauciullo, Sara Martorello, Kejsi Carku, Carmine Versace, Angelo Coletta & Gianfranco Cosenza

To cite this article: Alfredo Pauciullo, Sara Martorello, Kejsi Carku, Carmine Versace, Angelo Coletta & Gianfranco Cosenza (2021) A novel duplex ACRS-PCR for composite *CSN1S1–CSN3* genotype discrimination in domestic buffalo, Italian Journal of Animal Science, 20:1, 1264-1269, DOI: 10.1080/1828051X.2021.1952912

To link to this article: https://doi.org/10.1080/1828051X.2021.1952912

Published online: 15 Sep 2021.

ITALIAN JOURNAL OF ANIMAL SCIENCE
2021, VOL. 20, NO. 1, 1264–1269
https://doi.org/10.1080/1828051X.2021.1952912

Taylor & Francis
Taylor & Francis Group

SHORT COMMUNICATION

A novel duplex ACRS-PCR for composite *CSN1S1–CSN3* genotype discrimination in domestic buffalo

Alfredo Pauciullo[a], Sara Martorello[a], Kejsi Carku[a], Carmine Versace[a], Angelo Coletta[b] and Gianfranco Cosenza[c]

[a]Dipartimento di Scienze Agrarie, Forestali e Alimentari, University of Turin, Grugliasco, Italy; [b]Ricerca Innovazione e Selezione per la Bufala, Caserta, Italy; [c]Dipartimento di Agraria, University of Naples Federico II, Portici, Italy

ABSTRACT

This short communication aimed to develop a fast and straightforward method for the simultaneous discrimination in buffaloes of the alleles *CSN1S1* A/B and *CSN3* X1/X2 using a single protocol. DNA was isolated from 219 individual blood samples. A duplex artificially created restriction site (ACRS-PCR) was accomplished using two pairs of primers generating 86 bp (*CSN1S1*) and 160 bp (*CSN3*). Amplicons were contemporary digested by *Mbo*I and *Hin*fI for the identification of genotypes.

The double simultaneous amplification and digestion proved to be effective for allele identification. The method resulted particularly quick due to the small PCR amplicons and fast digest enzymes that allowed both a rapid amplification (about 1 h and 40 min) and digestion in 10 min. Population analysis indicated that minor allele frequencies were *CSN1S1* A (0.425) and *CSN3* X2 (0.306). Linkage disequilibrium (LD) showed r^2=0.46, and haplotype analysis revealed all four possible combinations with higher frequency for the *CSN1S1* B-*CSN3* X1 (0.553). Considering the tremendous economic impact of the *CSN1S1–CSN3* variants on the dairy production in buffalo, this method, applicable immediately after the birth from any DNA source, may speed up the selection of sires and dams' lines with more favourable genotypes.

HIGHLIGHTS

- *CSN1S1–CSN3* composite genotype influences dairy performances.
- Duplex ACRS-PCR identifies the *CSN1S1–CSN3* composite genotype in a single protocol.
- The duplex ACRS-PCR method may speed up the selection of lines with more favourable genotypes.

ARTICLE HISTORY
Received 22 March 2021
Revised 30 June 2021
Accepted 2 July 2021

KEYWORDS
River buffalo; αs1-casein; κ-casein; *CSN1S1*; *CSN3*; ACRS-PCR

Introduction

Caseins are the main proteins in ruminant milk and the influence of their genetic variants on milk composition and cheesemaking properties is well known to such an extent that sires selection takes into account their genotype at casein loci. In this respect, examples in cattle are represented by the variants of the κ-casein (*CSN3* A and B) for dairy processing milk or the alleles of the β-casein (*CSN2* A1 and A2) for drinking milk. The first has a quantitative effect on dairy yield, with the *CSN3**B variant reacting more promptly with rennet, having a curd coagulation time significantly shorter than milk with *CSN3**A so that sires with genotype *CSN3* BB are favourite. The latter influences the composition in bio-peptides derived by enzymatic digestion of the β-casein. The *CSN2**A1 variant releases the β-casomorphin-7 (BCM-7), an opioid affecting the gastrointestinal functions, including regulating motility and mucus production (Pal et al. 2015). Although the literature reports conflicting data about the BCM-7 effects, there is extensive evidence from animal trials and emerging evidence in humans that the BCM-7 is associated with slower gastrointestinal transit and hence increased gastrointestinal transit times (Brook-Taylor et al. 2017). In the last years, this result promoted the selection of sires with genotype *CSN2* A2A2. The interactions among variants of the complete casein cluster have also effects on production traits. For instance, the β-casein (*CSN2**B) together with the β-lactoglobulin (*LGB**B) were found to be

CONTACT Prof. Alfredo Pauciullo alfredo.pauciullo@unito.it Dipartimento di Scienze Agrarie, Forestali e Alimentari, University of Turin, Largo Paolo Braccini, Grugliasco 2-10095, Italy

more favourable for curd coagulation, firmness, and cheesemaking quality of milk (Di Stasio and Mariani 2000). In Italian Holsteins, Comin et al. (2008) found that *CSN3* and *CSN2* genotypes were strongly associated with milk coagulation traits and milk and protein yields, respectively. In particular, for coagulation time and curd firmness, the best *CSN2–CSN3* composite genotypes were those with at least one B allele at both loci (A1/B-A/B, A2/B-B/B, and A2/B-A/B), whereas for milk and protein yield, the best genotype was the most frequent genotype (A2/A2–A/A) (Comin et al. 2008).

Moreover, the composite genotypes *CSN1S1* BB-*CSN2* A2A2-*CSN3* BB showed the best coagulation characteristics and highest cheese yield in cattle (Perna et al. 2016; Albarella et al. 2020), so that composite genotype was proposed to be the most appropriate criterion for selection decisions.

Genetic variants associated with milk production (Pauciullo et al. 2012a, 2012b) or affecting the milk protein yield (Cosenza et al. 2015) are also known in river buffalo. In this respect, the occurrence of two DNA transversions at *CSN1S1* and *CSN3* might have a great economic impact on dairy yield and, consequently, on the selling price of buffalo milk mainly established by fat and protein content, and finally on sires selection for the genetic improvement of the breed. The first mutation, AJ005430:c.578C > T, realised at the 83rd nucleotide of the exon 17 of *CSN1S1* is responsible for the amino acid replacement p.Ser^{178}(B allele)/Leu^{178} (A allele) of the mature αs1-casein (Chianese et al. 2009). The *CSN1S1* B allele has been associated with higher protein content (Cosenza et al. 2015). The second transversion, HQ677596:c.536C > T, realised at the nucleotide 377 of the exon 4 of *CSN3* leads to the amino acid change p.Ile^{135}(X1 allele)/Thr^{135} (X2 allele) of the mature κ-casein (Mitra et al. 1998).

The effects of *CSN1S1-CSN3* composite genotypes of these variants on milk production traits and milk coagulation properties have been well described in the Mediterranean buffalo (Bonfatti et al. 2012, 2013). For instance, the genotype AA-X1X2 exhibited the shortest rennet coagulation time (RCT) and a similar effect was revealed for the curd-firming time (K_{20}). In addition to shortest RCT and K_{20}, genotype AA-X1X2 also revealed the largest curd firmness (A_{30}). Furthermore, recently Zicarelli et al. (2020) demonstrated that milk carrying *CSN1S1**B and *CSN3**X1 alleles resulted in a greater curd yield.

This short communication aimed to develop, for the first time, a simple and fast method for the simultaneous discrimination of the alleles *CSN1S1* A/B and *CSN3* X1/X2 in a single protocol. This method might be helpful for buffalo genotyping and animal selection independently from the milk production.

Material and methods

Sampling and DNA isolation

Sample collection was carried out on a total of 219 Italian Mediterranean river buffaloes belonging to one advanced dairy farm located in the Piedmont region (Northern Italy). Individual blood samples were collected during the routine prophylaxis of the farm by an official veterinarian of ASL (Local Sanitary Unit) of the Ministry of Health. For this reason, the Animal Care and Use Committee approval was not necessary.

Genomic DNA was isolated using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Concentrations and $OD_{260/280}$ ratios were measured with the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA).

Duplex ACRS-PCR and sequencing

The investigated mutations (*CSN1S1* AJ005430:c.578C > T and *CSN3* HQ677596:c.536C > T) do not alter recognition sites of endonuclease. Therefore, we decided to set up a duplex Artificially Created Restriction Site (ACRS)-PCR for allele discrimination. For this reason, two pairs of primers were designed: 5′-CAATACCCTGATGCCC**G**AT-3′ (*forward*) and 5′-CACCACAGTGGCATAGTAG-3′ (*reverse*) amplified a fragment of 86 bp at the *CSN1S1* (Cosenza et al. 2015), whereas 5′-TGTTGAGCCTACAAGTACAC**GA**A-3′ (*forward*) and 5′-GTTGTCTTCTTTGATGTCTCC-3′ (*reverse*) gave an amplicon of 160 bp for the *CSN3*. In this method, both forward primers were modified: the first by changing C→G to create the restriction site for *Mbo* I (↓GATC), and the second by replacing CT→GA to generate G↓ANTC restriction site for the *Hinf* I endonuclease.

Duplex PCR amplification was carried out in a final volume of 15 μL, including 50 ng of genomic DNA, 1× PCR Buffer (Promega, Madison, WI), 2.5 mM $MgCl_2$, 5 pmol of each primer, dNTPs 200 μM each, 1 U of *Taq* DNA Polymerase (Promega). The thermal conditions were: 95 °C for 4 min, 40 cycles at 95 °C for 30 s, annealing at 55 °C for 40 s, and extension at 72 °C for 20 s. A final extension was carried out at 72 °C for 5 min. The product specificity was confirmed by electrophoresis, loading 5 μL of PCR product on 3.0% agarose gel in 0.5× TBE buffer, stained with Sybr Green

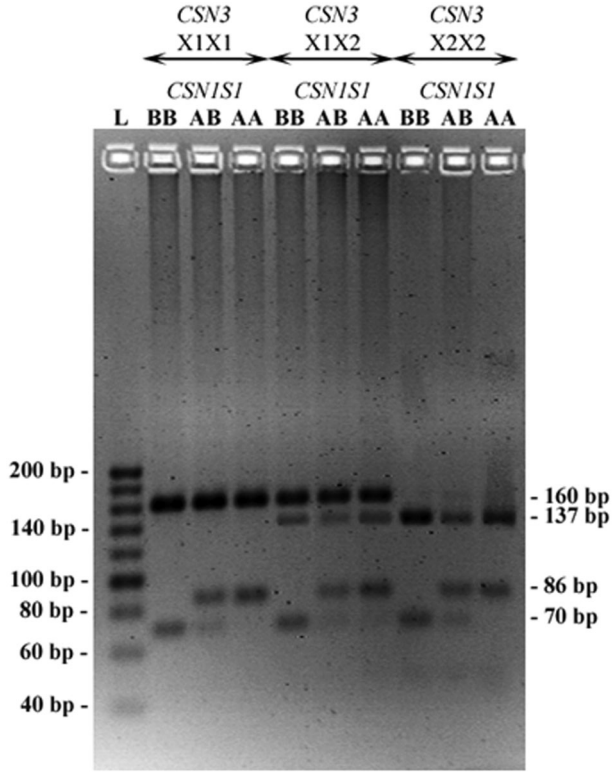

Figure 1. Simultaneous genotyping at the αS1- (*CSN1S1* alleles A and B) and κ-casein gene (*CSN3* alleles X1 and X2) by a duplex ACRS-PCR and following contemporary digestion with the *Mbo* I and *Hinf* I, in the Italian Mediterranean buffalo breed. L = 20 bp DNA ladder (20–200 bp log scale) (Jena Bioscience, Jena, Germany). The following three lanes are all *CSN3* X1X1 and *CSN1S1* BB, AB, AA, respectively. The lanes 5–7 are all *CSN3* X1X2 and *CSN1S1* BB, AB, and AA, respectively. The lanes 8–10 are all *CSN3* X2X2 and *CSN1S1* BB, AB, and AA, respectively.

(Lonza Rockland, Inc., Rockland, ME). The remaining 10 μL were contemporary digested with 1 μL of both Fastdigest *Mbo* I and *Hinf* I endonucleases (Thermo Fisher Scientific Inc.) for 10 min at 37 °C according to manufacturer's guidelines. The digested products were resolved on 3.5% agarose gel in 0.5× TBE buffer stained with Sybr Green for genotype detection.

For the validation and confirmation of the duplex ACRS-PCR genotype results, twelve informative samples (2 AA-X1X1, 3 AA-X2X2, 3 AB-X1X2, 3 BB-X1X1, 1 BB-X2X2) that resulted both double homozygous and heterozygous by ACRS-PCR were amplified in single PCR reactions and sequenced by Sanger Technology at Microsynth (Vienna, Austria).

Statistical analysis

Allele frequencies were calculated by Popgene software (University of Alberta, Edmonton, Alberta, Canada), whereas Phase 2.1 (Stephens and Scheet 2005) was used to determine the haplotype frequencies, and Haploview 4.2 (Barrett et al. 2005) was used to define linkage disequilibrium.

Results and discussion

Domestic buffalo is an expanding breeding reality worldwide. This is mainly due to the rheological characteristics of its milk, known to have higher protein and fat content compared to other ruminant's milk. These properties make it particularly suitable for the cheesemaking with a higher yield production (Zicarelli 2004; Zicarelli et al. 2020). Despite the potential economic impact on the dairy industry, increasing buffalo milk production with optimal percentages of fats and proteins through an adequate genetic selection process still reveal delays. This is also due to the lack of fast methods for the discrimination of favourable alleles for more profitable breeding strategies, such as the A/B and X1/X2 alleles at αs1- and κ-casein genes, respectively (Bonfatti et al. 2012; Bonfatti et al. 2013; Cosenza et al. 2015; Zicarelli et al. 2020).

To date, RP-HPLC of milk protein and/or DNA sequencing were the most used methods for genotype determination at *CSN1S1* and *CSN3* genes (Chianese et al. 2009; Bonfatti et al. 2012; Bonfatti et al. 2013; Zicarelli et al. 2020). However, both methods require several working steps and have limitations. For instance, RP-HPLC cannot be used for sires genotyping, whereas sequencing needs the development of separated PCR protocols, purifications, formamide treatments before sequencing, etc. To overcome these restrictions, in this study, we developed for the first time a duplex ACRS-PCR method followed by a digestion for the contemporary discrimination of *CSN1S1* A/B and *CSN3* X1/X2 alleles.

The double simultaneous amplification and digestion proved to be effective for genotype identification (Figure 1). For *CSN1S1* AJ005430:c.578C > T, the digestion of the PCR product (86 bp) with the *Mbo* I endonuclease produced two fragments (70 and 16 bp) for the presence of the cytosine (allele B), and undigested fragment (86 bp) for the presence of the thymine (allele A). Simultaneously, for the SNP *CSN3* HQ677596:c.536C > T, using the endonuclease *Hinf* I, in presence of the cytosine (allele X1), the fragment remained undigested (160 bp), whereas in the occurrence of the thymine (allele X2), two fragments (137 and 23 bp) were obtained (Figure 1).

The method resulted particularly fast due to the small PCR amplicons and the use of fast digest enzymes that allowed both a rapid amplification

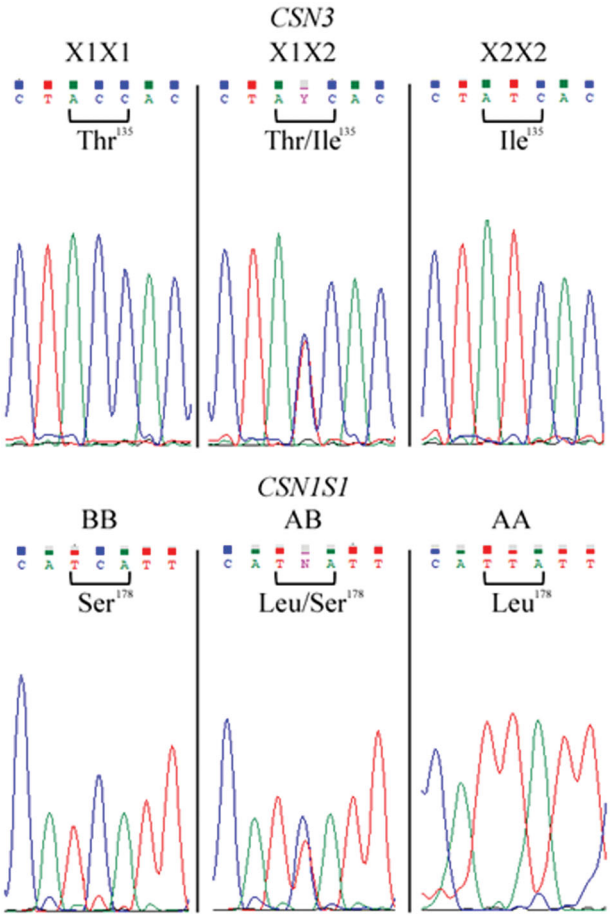

Figure 2. Sanger sequencing of the informative DNA samples. Double homozygous and double heterozygous at *CSN1S1* and *CSN3* *loci* as confirmation of the developed genotyping method.

(about 1 h and 40 min) and the digestion after 10 min of incubation time only. Sanger sequencing was performed for the validation of the method. Electropherogram analysis confirmed the result of the genotyping by ACRS-PCR (Figure 2). However, the whole procedure (set up of two amplification protocols, PCR purification, sequencing reaction, sequencing run) resulted in time-consuming compared to the method proposed herein, and more expensive in terms of costs. We assessed a saving of 75% for the costs sustained with our method versus the sequencing. However, we do not exclude a certain range of saving, depending on the market evolution, country, buyers–sellers agreements for reagents' orders, etc.

Genotyping data indicated that minor allele frequencies were 0.425 for the *CSN1S1* A and 0.306 for the *CSN3* X2 (Table 1). Our results agree with the data produced by Bonfatti et al. (2012) and Zicarelli et al. (2020). However, in this respect, it must be underlined that in the two quoted papers (Bonfatti et al. 2012; Zicarelli et al. 2020) the allele nomenclature for both *loci* was reversed in comparison to the original one (Mitra et al. 1998; Chianese et al. 2009) that we considered as reference.

LD analysis performed with our data showed r^2=0.46, and haplotype analysis revealed all four possible combinations with higher frequency for the *CSN1S1* B-*CSN3* X1 (Table 1). Conversely, a high linkage disequilibrium ($p < .001$) between *CSN1S1* and *CSN3* genes were found by Bonfatti et al. (2012). However, this is a relatively rare event. In fact, it is well-known that many recombination events characterise the casein cluster (Caroli et al. 2009; Pauciullo et al. 2019).

The most favourable haplotype for dairy purposes observed in our study (*CSN1S1* A-*CSN3* X2 according to the original nomenclature) showed a frequency of 0.279. According to Zicarelli et al. (2020), buffaloes carrying these casein variants produced a greater curd yield due to higher proteins rather than milk production, and greater real curd yield/estimated curd yield ratio (RCY/ECY).

Considering the relatively low frequency of this haplotype (*CSN1S1* A-*CSN3* X2), the choice of animals with better dairy performances and the consequent indirect selective pressure applied in this advanced buffalo farm, whose exclusive attitude is dairy production, might leave very wide opportunities of genetic progress.

Conversely, the most frequent haplotype (0.553) observed in the present study was *CSN1S1* B-*CSN3* X1. According to the original nomenclature, this haplotype corresponds to a lower curd yield (Zicarelli et al. 2020). In general, the negative effect on the dairy characteristics was mainly attributed to the *CSN3* X1 allele, indicated as the worst for the RCY/ECY ratio, independently from its association with any *CSN1S1* genotype (Zicarelli et al. 2020).

Although the results refer to a single herd and should be tested population-wide, it is of great importance as the method appeared effective for the recognition of composite *CSN1S1* and *CSN3* genotypes in domestic buffalo, one of the most relevant dairy traits for its influence on milk production and cheese-making properties.

In conclusion, a new very fast protocol based on a single ACRS-PCR was set up for the simultaneous genotyping of *CSN1S1* A/B-*CSN3* X1/X2 alleles, known to have significant effects on dairy performances of river buffaloes. The protocol minimises the cost of analyses on a large scale (up to 75% cheaper than the sequencing) and gives most laboratories with basic equipment and average economic resources to perform this assay. Furthermore, considering the great

Table 1. Genotypes, allele, and haplotype frequencies detected at the *CSN1S1* and *CSN3* genes in the Italian Mediterranean river buffalo breed.

	Genotypes				Allele frequencies				Allele frequencies		
	CSN3				*CSN1S1*		*CSN3*			Frequency	SE
	X1X1	X1X2	X2X2	Total	A	B	X1	X2	BX1	0.553	0.0024
CSN1S1											
AA	2 (0.91)	14 (6.39)	27 (12.33)	43 (19.63)	0.425	0.575	0.694	0.306	AX2	0.279	0.0024
AB	43 (19.63)	56 (25.58)	1 (0.45)	100 (45.66)					AX1	0.145	0.0024
BB	69 (31.50)	6 (2.73)	1 (0.45)	76 (34.70)					BX2	0.023	0.0024
Total	114 (52.04)	76 (34.70)	29 (13.25)	219 (100.00)							

economic impact on dairy production, this method, applicable immediately after the buffalo birth from any source of DNA, may speed up the selection of sires and dams' lines with more favourable genotypes.

Ethical approval

All procedures observed the Directive 98/58/EC concerning the protection of animals. Sampling has been carried out during the routine prophylaxis of the farm by an official veterinarian of ASL (Local Sanitary Unit) of the Ministry of Health. Therefore, according to the Committee on the Ethics of Animal Experiments of the University of Torino (D.R. n. 2128 released on 06/11/2015) further ethics approval was not required.

Acknowledgements

The authors thank Morisiasco Ivan and Moris farm in the Piedmont region for providing river buffalo samples.

Disclosure statement

The authors declare no conflict of interest with any financial organisation regarding the material discussed in the manuscript.

Funding

This work was partially supported by the Italian Ministry of Research [Project no. PON01_00486 GENOBU] and by Ricerca Innovazione e Selezione per la Bufala [Project no. PAUA_RIC_N_COMP_20_02].

ORCID

Alfredo Pauciullo http://orcid.org/0000-0002-3140-9373
Gianfranco Cosenza http://orcid.org/0000-0001-6006-4987

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

References

Albarella S, Selvaggi M, D'Anza E, Cosenza G, Caira S, Scaloni A, Fontana A, Peretti V, Ciotola F. 2020. Influence of the casein composite genotype on milk quality and coagulation properties in the endangered Agerolese cattle breed. Animals. 10(5):892.

Barrett JC, Fry B, Maller J, Daly MJ. 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 21(2):263–265.

Bonfatti V, Gervaso M, Rostellato R, Coletta A, Carnier P. 2013. Protein composition affects variation in coagulation properties of buffalo milk. J Dairy Sci. 96(7):4182–4190.

Bonfatti V, Giantin M, Gervaso M, Coletta A, Dacasto M, Carnier P. 2012. Effect of CSN1S1-CSN3 (α(S1)-κ-casein) composite genotype on milk production traits and milk coagulation properties in Mediterranean water buffalo. J Dairy Sci. 95(6):3435–3443.

Brook-Taylor S, Dwyer K, Woodford K, Kost N. 2017. Systematic review of the gastrointestinal effects of A1 compared with A2 β-casein. Adv Nutr. 8(5):739–748.

Caroli A, Chessa S, Erhardt G. 2009. Invited review: milk protein polymorphisms in cattle: effect on animal breeding and human nutrition. J Dairy Sci. 92(11):5335–5352.

Chianese L, Quarto M, Pizzolongo F, Calabrese MG, Caira S, Mauriello R, De Pascale S, Addeo F. 2009. Occurrence of genetic polymorphism at the αs1-casein locus in Mediterranean water buffalo milk. Int Dairy J. 19(4): 181–189.

Comin A, Cassandro M, Chessa S, Ojala M, Dal Zotto R, De Marchi M, Carnier P, Gallo L, Pagnacco G, Bittante G. 2008. Effects of composite beta- and kappa-casein genotypes on milk coagulation, quality, and yield traits in Italian Holstein cows. J Dairy Sci. 91(10):4022–4027.

Cosenza G, Pauciullo A, Macciotta N, Apicella E, Steri R, La Battaglia A, Jemma L, Coletta A, Di Berardino D, Ramunno L. 2015. Mediterranean river buffalo CSN1S1 gene: search for polymorphisms and association studies. Anim Prod Sci. 55(5):654–660.

Di Stasio L, Mariani P. 2000. The role of protein polymorphism in the genetic improvement of milk production. Zootecn Nutri Anim. 26:69–90.

Mitra A, Schlee P, Krause I, Blusch J, Werner T, Balakrishnan C, Pirchner F. 1998. Kappa-casein polymorphisms in Indian dairy cattle and buffalo: a new genetic variant in buffalo. Anim Biotechnol. 9(2):81–87.

Pal S, Woodford K, Kukuljan S, Ho S. 2015. Milk intolerance, beta-casein and lactose. Nutrients. 7(9):7285–7297.

Pauciullo A, Cosenza G, Steri R, Coletta A, Jemma L, Feligini M, Di Berardino D, Macciotta NP, Ramunno L. 2012a. An association analysis between OXT genotype and milk yield and flow in Italian Mediterranean river buffalo. J Dairy Res. 79(2):150–156.

Pauciullo A, Cosenza G, Steri R, Coletta AL, Battaglia A, Di Berardino D, Macciotta NP, Ramunno L. 2012b. A single nucleotide polymorphism in the promoter region of river buffalo stearoyl CoA desaturase gene (SCD) is associated with milk yield. J Dairy Res. 79(4):429–435.

Pauciullo A, Shuiep ET, Ogah MD, Cosenza G, Di Stasio L, Erhardt G. 2019. Casein gene cluster in camelids: comparative genome analysis and new findings on haplotype variability and physical mapping. Front Genet. 10:748.

Perna A, Intaglietta I, Gambacorta E, Simonetti A. 2016. The influence of casein haplotype on quality, coagulation, and yield traits of milk from Italian Holstein cows. J Dairy Sci. 99(5):3288–3294.

Stephens M, Scheet P. 2005. Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. Am J Hum Genet. 76(3):449–462.

Zicarelli L. 2004. Buffalo milk: its properties, dairy yield and mozzarella production. Vet Res Commun. 28:127–135.

Zicarelli L, Di Palo R, Napolano R, Tonhati HD, Carlo E, Gagliardi R, Di Luccia A, la Gatta B. 2020. Influence of αS1-casein and κ-casein polymorphism on the curd yield of Italian Mediterranean buffalo (Bubalus bubalis L.) milk. Int Dairy J. 100:104559.