Remarkable genetic diversity detected at river buffalo prolactin receptor (PRLR) gene and association studies with milk fatty acid composition

Remarkable genetic diversity detected at river buffalo prolactin receptor (*PRLR*) gene and association studies with milk fatty acid composition

G. Cosenza,* M. Iannaccone,*,1 B. Auzino,† N.P.P Macciotta,‡ A. Kovitvadhi,§ I. Nicolae,# and A. Pauciullo ‖

* Department of Agricultural Sciences, University of Naples "Federico II", 80055, Portici (NA), Italy

† Department of Veterinary Sciences, University of Pisa, 56124, Pisa, Italy

‡ Department of Agricultural Sciences, University of Sassari, 07100, Sassari, Italy

§ Department of Physiology, Kasetsart University, 10900, Bangkok, Thailand

Research and Development Institute for Bovine Breeding, 077015, Balotesti, Bucharest, Romania

‖ Department of Agricultural, Forest and Food Science, University of Torino, 10095, Grugliasco (TO), Italy

1 Corresponding author: m.iannaccone@unina.it

Summary

Prolactin is an anterior pituitary peptide hormone involved in many different endocrine activities and is essential for reproductive performance. This action is mediated by its receptor, the prolactin receptor, encoded by the *PRLR* gene. In this study, we sequenced and characterized the Mediterranean river buffalo *PRLR* gene (from the exon 3 to 10) and a remarkable genetic diversity was found. In particular, we found 24 intronic polymorphisms and 13 exonic SNPs, 7 of which were non-synonymous. Furthermore, the polymorphisms identified in the 3'UTR were investigated to establish their possible influence on microRNA binding sites. Considering all the amino acid changes and the observed allelic combinations, it is possible to deduce at least 6 different translations of the buffalo prolactin receptor and, consequently, the presence at the *PRLR* gene of at least 6 alleles. Furthermore, we identified a deletion of a CACTACC heptamer between the nucleotides 1102-1103 of exon 10 (3'UTR) and we developed an allele specific PCR to identify the carriers of this genetic marker.

Finally, the SNP g.11188A>G, detected in exon 10 and responsible for the amino acid replacement p.His328Arg, was genotyped in 308 Italian Mediterranean river buffaloes and an association study with milk fat traits was carried out. The statistical analysis showed a trend that approached the significance for the AA genotype with higher contents of odd branched-chain fatty acids (OBCFA). Thus, our results suggest that *PRLR* gene is a good candidate for gene association studies with qualitative traits related to buffalo milk production.

Keywords: Mediterranean river buffalo, *PRLR*, alleles, association, milk, OBCFA

Introduction

Prolactin (encoded by *PRL* gene) is peptide hormone produced by anterior pituitary and it plays a role in many different endocrine activities and is essential for reproductive performance. These actions are mediated by the prolactin receptor, which has been detected in various tissues including brain, ovary, placenta and uterus of several mammalian species. The prolactin receptor, encoded by *PRLR* gene, belongs to the growth hormone/prolactin receptor gene family sharing high homology sequence regions.

In cattle, *PRLR* gene is mapped on chromosome 20 (20q17) (Hayes *et al*., 1996). It is made by 10 exons and 3 of them can be alternative spliced with at least 2 different known isoforms, long and short (Lü *et al*., 2011). Both the isoforms are differentially expressed and regulated during estrous cycle and pregnancy, indicating a role in different metabolic pathways (Buck *et al*., 1992; Clarke *et al*., 1993, Clarke & Linzer 1993). In particular, the long isoform of *PRLR* binds *PRL* and contributes to activation of the transcription factor JAK2 (janus kinase 2) and subsequent phosphorylation of STAT5 (signal transducer and activator of transcription 5), which recognize sequences located in promoters of milk protein genes (Bole-Feysot *et al*. 1998). In ruminants, the long and short isoforms encode for a protein of 581 and 296 amino acids, respectively. The short protein is originated by an alternative splicing of 39 bp (in the exon 10) that generate 2 close stop codons (Bignon *et al*. 1997; Lü *et al*., 2011).

Several studies have highlighted the influence of *PRLR* on the reproductive performance in sheep and pig (Tomás *et al*. 2006; Chu *et al*., 2007), the induction of maternal behavior in sheep (Wang *et al*., 2015) and the litter size in goat (Hou *et al*., 2015). Moreover, *PRLR* expression is important during mouse mammary gland development (Bole-Feysot *et al*. 1998).

In cattle, quantitative trait *locus* (QTL) on chromosome 20 and *PRLR* gene polymorphisms have been associated with milk production traits (Zhang *et al*. 2008; Iso-Touru *et al*. 2009, Lü *et al*., 2011), as well as in pig (Skrzypczak *et al*., 2015) and sheep (Hou *et al*., 2013, 2014). Based on these

studies, *PRLR* gene is considered as a strong candidate for gene association studies with quantitative and qualitative traits related to milk production.

Recently, different studies have shown that the *PRLR* gene is polymorphic also in *Bubalus bubalis* (mainly Murrah river buffalo) (Javed *et al*., 2011a,b; Shi *et al*., 2012), but to date none of the polymorphisms identified have been investigated for association with milk production traits.

Therefore, the aim of the present research was to identify and analyse the variability of the *PRLR locus* in Mediterranean river buffalo reared in Italy and test the possible associations between polymorphisms and milk fat traits in order to contribute to the improvement of the productive efficiency of the breed.

Materials and Methods

Sample collection DNA isolation and PCR amplification conditions

Individual blood samples were collected from 308 female Mediterranean river buffaloes belonging to 14 farms, located in Salerno and Caserta province (Southern Italy) plus 150 samples collected from Romanian Murrah (100) and Thai Mehsana (50). Sampling of Italian river buffaloes was carried out in collaboration with the Italian National Association of Buffalo Breeders (ANASB).

DNA was isolated from leukocytes, using the procedure described by Goossens & Kan (1981). DNA concentration and $OD_{260/280}$ ratio of the samples were measured by the Nanodrop ND-2000C Spectrophotometer (Thermo Scientific).

Using primers designed on bovine (GenBank acc. no. AF426315.1), bubaline genome sequence (NW_005785465) and bubaline mRNA sequence (GenBank acc. no. GQ339914.1), we amplified the DNA region of the *PRLR* gene spanning from the exon 3 to the exon 10 in ten individual Mediterranean river buffaloes reared in 10 different farms. Additional primers, designed on newly determined sequences, were also used for sequencing. PCR products were sequenced on both strands at CEINGE - Biotecnologie Avanzate (Naples, Italy).

The estimation of SNPs frequency at exon 10 and the determination of possible haplotypes was accomplished by the re-sequencing of additional 48 and 10 samples of Mediterranean and Mehsana breeds, respectively. All sequenced samples were randomly selected.

PCR Reaction mix and thermal condition are reported in supplementary materials.

The entire panel of 308 Mediterranean river buffalo DNA samples was genotyped in outsourcing (KBiosciences, Herts, UK, http://www.kbioscience.co.uk) for the SNP g.11188A>G (Nuova Tabella)

Allele Specific-Polymerase Chain Reaction (AS-PCR) for the heptamer detection

An allele-specific PCR (AS-PCR) was developed to identify carriers of the CACTACC heptamer between nucleotides 1102 and 1103 of exon 10 (3'UTR) of *PRLR* gene in river buffalo. The two allele-specific reverse primers differ for absence (5'-AACTGGGAGTTGTCATTCTAA-3') or presence (5'-AACTGGGAGTTGGGTAGTG-3') of the sequence corresponding to the insertion (herein underlined). The allele-specific reverse primers and the forward common primer sequence (5'-CTCTGCTAAACCCTTGGAAT-3') are part of the exon 10. Primers were designed according to the newly determined sequences and by the comparison with the corresponding goat (XM_018065467.1), sheep (KC734660.1) and bovine (AJ966356.4) sequences. Reaction mix and thermal condition were performed as reported above.

Genotyping was carried out on buffaloes belonging to the Italian Mediterranean (300), Murrah (100) and Mehsana (50) buffalo breeds, as well as cattle (50), sheep (50) and goats (50) belonging to different breeds/genetic types randomly chosen (Nuova Tabella).

Bioinformatic and statistical analysis

Allelic frequencies and Hardy-Weinberg equilibrium ($\chi 2$ test) were calculated. Homology searches, comparison among nucleotide and amino acid sequences, multiple alignments for polymorphism discovery were accomplished using DNAsis-Pro (Hitachi Software Engineering Co., Japan).

Measures of linkage disequilibrium (D' and r^2) were estimated using Haploview software ver. 4.2 (http://www.broadinstitute.org/haploview/haploview). Haplotype structure was defined according to Gabriel *et al*. (2002).

Associations between *PRLR* genotype, fatty acid composition traits, fatty acid classes and fat percentage were tested using a mixed linear model (Supplementary materials).

MicroRNAs screening was performed using TargetScan (Lewis *et al*., 2005) based on homology between our sequence and the bovine 3'UTR of *PRLR* gene.

Results and Discussion

Structure and analysis of Mediterranean river buffalo PRLR gene

Using genomic DNA from 10 Mediterranean river buffaloes reared in Campania, we sequenced the coding region of prolactin receptor gene (GeneBank accession number MF461277).

The sequenced region (over 11900 bp, including 1138 bp of exonic regions) spans from the exon 3 to the exon 10 (excluding mostly of intron 5, about 12000 bp). The sequenced *PRLR* exons and introns have different length: from 70-100 bp for the exons 9 and 8 to over 1180 bp for the exon 10, while introns start from 693 bp (intron 8) to 2756 bp (intron 3). Splicing acceptor and donor sites followed the rule 5' GT/ 3'AG (Figure S1). The homology with the bovine counterpart is 95% with a G/C content of 45%.

Signal peptide is 24 amino acid long (MKENAASRVVFILLLFLNFSLLNG) and it is encoded by the last 70 nt of exon 3 and the first 2 nt of exon 4. The stop codon (TGA) is localized between the nt 873-875 of the exon 10, which encodes for the majority of intracellular domain. This structure is highly conserved among mammals and, depending on the size of cytoplasmic domain, two mRNAs named short and long have been described (Bignon *et al*., 1997; Zhang *et al*., 2008). The only difference between short and long coding sequences was the presence or absence, respectively, of an insert 39 bp long at the beginning of the cytoplasmic domain. The insertion creates two contiguous *in frame* stop codons at its 3' end. As described by Bignon *et al*. (1997), this insertion,

present also in Mediterranean river buffalo (Figure S1), is an alternative splicing strategy generated by the use of a splice donor site at 40–41 bp upstream the exon 10. As consequence, the short and long form are 296 aa and 581 aa, respectively.

Intronic regions analysis and polymorphisms detection in Mediterranean river buffalo

Alignments of the intron sequences among the 10 subjects showed 24 polymorphisms (6 transversions, 15 transitions and 3 deletions/insertions) (Table 1) and several microsatellites that were not polymorphic in these samples. However, none of these polymorphisms would seems to affect expression regulation sequences (splicing sites, enhancers/silencers).

As it was already demonstrated for several ruminant (Ramunno *et al*., 2004) and non-ruminant species (Pauciullo *et al*., 2013; Pauciullo *et al*., 2014), also the buffalo *PRLR* gene is characterized by the presence of several retroposons located in the introns and representing about 13% of the entire gene.

According to Lenstra *et al*. (1993), three different types of artiodactyla retroposons have been described. In particular, we have identified 6 retroposon sequences: A, B, C, D, E and F in buffalo *PRLR* gene (Figure S1). The elements A and B are localized in the intron 4. In particular, sequence A (between nt 1338 and 1600, taking as reference the first nucleotide of the corresponding intron) is a Bov-A2 element-like, while the sequence B (from nt 1648 to nt 1880) shows high homology with the retroposon Bov-tA2. The element C is located in the intron 6 (Bov-tA2 like, from 457 to 678), while the sequence D is in the intron 7 from nt 388 to nt 725 (Bov-B like). The last elements E (Bov-tA2, from 317 to 496) and F (Bov-A2, between nt 241 and 522) are located in the introns 8 and 9 respectively. The elements A, B, C and F are flanked by imperfect direct repeats, underlined in Figure S1. All the aforementioned retroposons are well conserved among ruminants for which genomic sequences are available (homology higher than 94%).

Exon polymorphism analysis in Mediterranean and Mehsana

168 The alignment of *PRLR* exons from the 10 investigated subjects of Mediterranean river buffalo
169 identified 13 polymorphisms, 7 of which were non-synonymous SNP. In particular, the first two of
170 the 13 polymorphisms identified, are located at the exon 3 and 7 respectively, while the remaining at
171 10^{th} exon (Table 1). Two of these mutations (g.114T>G and g.11188A>G, responsible for the aa
172 changes p.Phe19Val and p.His328Arg, respectively) have already been reported in Indian water
173 buffalo breeds by Javed *et al*. (2011a).

174 Moreover, the comparison between the sequences available in GenBank for the Mediterranean
175 river buffalo (GenBank NW_005785465, XR_328736.1, XM_006071901.1, XM_006071900.1)
176 pointed out other two polymorphic sites in exon 10: g.11465T>C and g.11563C>T, the latter
177 responsabile for the amino acid change p.Ser453Leu. All the buffaloes investigated were
178 monomorphic for the last two *loci*,

179 Beside the polymorphisms observed for the Mediterranean buffalo, the sequencing of the exon
180 10 in ten Mehsana samples showed a new non-synonymous SNP: g.11206C>T responsible for the
181 amino acid change p.Thr334Met (Table SX).

182 Another non-synonymous mutation at the exon 10 of the *PRLR* gene has been described by
183 Javed *et al*. (2011b) for Indian water buffalo breeds (Murrah and Mehsana): the transition
184 g.11488A>G responsible for the amino acid replacement p.Ser428Gly. However, this polymorphism
185 was not observed in any of the subjects examined in the present study.

186 Based on the comparison with the sequences available in GenBank for the main species
187 belonging to the order Cetartiodactyla and Perissodactyla (Table Sx), at least 7 polymorphisms
188 (g.114T>G, g.11188A>G, g.11206C>T, g.11563C>T, g.11687A>G, g.11882G>A, g.12100A>G)
189 showed particular variability among the different mammalian species, so that haplotypes specific of
190 specie/order/suborder can be identified .

191 Furthermore, comparing our sequences with those reported in Table Sx, two polymorphisms
192 at the buffalo *PRLR locus* also characterize other species of ruminants and, therefore they may
193 represent possible examples of trans-specific polymorphisms (TSPs).

The first of these mutations (g.11188A>G responsible for the change p.His328Arg) also characterizes the *PRLR locus* in goat: C<u>G</u>C^{Arg328} (GenBank HQ667935, NM_001285669); C<u>A</u>C^{His3288} (GenBank KC906265, JX087440). The second polymorphism (g.11936G>A, p.Pro577) was also reported in cattle (FJ901289; Iso-Touru *et al*., 2009).

Moreover, the comparison with the sequences available in GenBank shows a further possible TSP for the *PRLR locus* which, however, affects only the ovine and caprine species. This is a silent transition A>G in position 276 of 10th exon (Pro^{377}): goat A^{276} (GenBank EU662222, EU662223), goat G^{276} (GenBank NM_001285669, JX087440), sheep A^{276} (GenBank FJ901301, AF041979), sheep G^{276} (GenBank HM437209, HM437208).

TSPs are ancient genetic variants whose origin predates speciation events, resulting in shared alleles between evolutionarily related species (Klein *et al*., 1987). TSPs can be explained by three main reasons: 1) SNP survival due to random chance, 2) coincidental mutations occurred after speciation and 3) balancing selection. TSPs as striking signature of persistent balancing selection are not very common (Hedrick, 2006). Examples of trans-specific polymorphisms have been reported in different species, including humans. To date, putative TSPs between humans and other primate species have been identified for the highly polymorphic major histocompatibility complex (MHC), the histo-blood ABO group, two antiviral genes (ZC3HAV1 and TRIM5), an autoimmunity-related gene LAD1 and several non-coding genomic segments with a putative regulatory role (Azevedo *et al*., 2015). On contrary, there are very few documented examples of TSPs in ruminants. Only recently, Cosenza *et al*. (2017a) detected a first possible example of TSPs in the oxytocin-neurophysin I hormone encoding gene (*OXT*) in goat and sheep. Therefore, the identification of the detected polymorphisms could represent a new report of TSPs at DNA-level in ruminants and the first in Mediterranean river buffalo.

Moreover, the further comparison of the obtained sequences and the sequences available in database for the main ruminant species shows for the Mediterranean and Mehsana breeds the deletion of the CACTACC heptamer between the nucleotides 1102 and 1103 of 10th exon (3'UTR).

The presence of the heptamer might represent the ancestral condition of the gene, because it has also been found in the main non-ruminant species belonging to order Cetartiodactyla and in Perissodactyla (Figure S2). In particular, the members of suborder Tylopoda and Suiformes are characterized by a similar molecular event: CACTACCA and CACTACCC insertions, respectively, while members of suborder Hippomorpha and Ceratomorpha (order Perissodactyla) are characterized by CACTACTA (Figure S2).

In order to verify the specificity of this genetic marker, an allele specific PCR method was developed. By means of this method, the *PRLR* homozygote samples for the insertion showed successful PCR amplification (462 bp) only using the reverse primer for the "insertion". Conversely, the homozygote samples for "non-insertion" allele (455 bp) are successfully amplified only by reverse primer with no insertion.

The genotyping of buffaloes from Mediterranean (300), Murrah (100) and Mehsana (50) breeds (all belonging to the river type) in comparison to cattle (50), sheep (50) and goats (50) belonging to different breeds/genetic types randomly chosen, confirmed that only our buffaloes samples (450) are characterized by the absence of the heptamer (Figure 1). However, our results from Murrah and Mehsana breeds conflict with a *PRLR* genomic sequence deposited in GenBank (GQ339914.1) by Javed *et al*. (2011) for Indian buffalo breeds (Murrah, Bhadawari, Tarai, Pandharpuri, Marathwada and Mehsana). Considering the conflicting information, we can not claim that the eptamer deletion is peculiar also of Murrah and Mehsana breeds, but we can surely state that it is specific for the Mediterranean breed.

Observed haplotypes at the river buffalo PRLR locus

In order to estimate the frequency of the 11 polymorphic sites identified at the exon 10 of the Mediterranean river buffalo *PRLR* gene and to determine the possible haplotypes, the entire exon was sequenced a total of 68 samples (58 Mediterranean subjects reared in Southern Italy and 10 Mehsana buffaloes reared in Thailand) (Nuova tabella).

Three different allelic combinations were observed: haplotype 1, 2 and 3. Of these, the haplotype 1 is the most represented with a frequency of 0.62, followed by the haplotype 2 (0.25) and 3 (0.13) (Figure 2).

According to Gabriel *et al*. (2002), the first 9 SNPs of the exon 10 (position 11188, 11434, 11577, 11580, 11683, 11687, 11768, 11882, 11936) can be considered as belonging to one haplotype block, whereas the *loci* 12014 and 12100 constitute a second haplotype block (Figure 2).

To these haplotypes it is possible to add the three haplotypes inferred from sequences available in the GenBank for the same breed (haplotype 4, GenBank XR_328736, XM_006071901, NW_005785465 and XM_006071900) and for the buffalo reared in India (haplotypes 5 and 6, GenBank HQ236497, GQ339914) (Table S2).

Therefore, taking into consideration all the 7 amino acid changes (exons 3, 7 and 10) and the observed allelic combinations, it is possible to deduce at least 6 different possible translations of the buffalo prolactin receptor and, consequently, the presence at this *locus* of at least 6 alleles (*PRLR* A, B, C, D, E and F, Figure 3).

The SNPs in position 11465 and 11563 were excluded from this analysis because they were identified only by the comparison of the sequences available in GenBank for Mediterranean (GenBank XR_328736, XM_006071901, NW_005785465 and XM_006071900) and Mehsana breeds (present work) (Table S1).

The high level of variability found at the exon 10 of the *PRLR* buffalo gene, which represents most of the intracellular domain, is probably related primarily to the alternative splicing of the primary transcript relative to this exon (Trott *et al*., 2003; Tomás *et al*., 2006), suggesting that this particular DNA region (coding) can accumulate a significant amount of polymorphisms, similar to that observed in other species.

The analysis of polymorphisms at the *PRLR locus* in European cattle (*Bos taurus*), African (*Bos indicus*), yak (*Bos grunniens*), and bison (*Bison bison*) has, in fact, revealed a comparable degree

of variability and the existence of several (at least 20) haplotypes (Iso-Touru *et al*., 2009). A similar level of genetic diversity was described also at *PRLR* locus in pig (*Sus scrofa*) (Tomás *et al*., 2006).

Fatty acids composition profile, genotyping and association of PRLR polymorphism with milk FA composition traits

Fatty acid composition, fatty acid classes and fat percentage of milk samples have been assessed and previously reported by Cosenza *et al*. (2017b). The same phenotypes were also used in the present work to assess possible associations with the genetic diversity found at the *PRLR*. Since the haplotype analysis evidenced that most of SNP found at the exon 10 belong to one haplotype block, only one SNP (g.11188A>G) was genotyped and considered in running model.

The genotyping of 308 buffalo showed that major allele (adenine) had a relative frequency of 0.9 and the $\chi 2$ value showed that there was no evidence of departure from the Hardy-Weinberg equilibrium ($P \leq 0.05$). Genotype distribution of buffalo cows for the SNP g.11188A>G is reported in Table 2.

Results of the model (Supplementary materials) showed a tendency that approached a significance effects ($P<0.06$) of the SNP g.11188A>G (AA genotype) at the *PRLR locus* on the milk FA composition traits with higher contents of odd branched-chain fatty acids (OBCFA), belonging to polyunsaturated FA class (Table S3). OBCFA acids are an emerging class of bioactive FA that reduce the incidence of necrotizing enterocolitis in newborns, have anti-tumor effects on lymphomas, and improve pancreatic β-cell function. However, the interest in OBCFA arises from their anticarcinogenic effects on cancer cells comparable to that of conjugated linoleic acid (Bainbridge *et al*., 2016). OBCFA acids are typically of exogenous origin, deriving from forages or supplements given to the animal and from metabolism of bacteria living in the rumen, respectively. Because OBCFA are constituents of rumen bacterial membranes (Kaneda, 1991; Mackie *et al*., 1991), dairy and meat products from ruminants are a unique source of these FA. This allows for OBCFA to be

used as biomarkers of dairy intake in humans and also as biomarkers of rumen function in cattle (Stefanov *et al*., 2010).

It is possible to hypothesize that the SNP g.11188A>G may be in linkage disequilibrium with another mutation in *PRLR* gene affecting a greater extent yield traits rather than being causal. For example, interrogating the software targetscan (Lewis *et al*., 2005) looking for microRNA targeting the bovine *PRLR* (ENST00000310101.5) and based on the high homology between *Bos taurus* and *Bubalus bubalis*, we found that g.12100A>G influences binding of miR-125b (Figure S3) and, potentially, affect *PRLR* expression.

The studies carried out to date in bovine, caprine and porcine species have already detected different polymorphisms at *PRLR locus* and many of these were significantly associated with phenotypic traits related to milk production, in particular, milk yield and fat content (Zhang et al. 2008; Iso-Touru *et al*. 2009, Lü *et al*., 2011; Hou *et al*., 2013, 2014; Skrzypczak *et al*., 2015).

Our results confirm the influence of *PRLR* gene on buffalo milk acidic composition and, consequently, milk quality, particularly the mutations that fall into the exon 10 of the gene. In fact, it was suggested that the *PRLR* long form plays a more important role in the regulation of the lactation (Zi *et al*., 2012). In particular, the up-regulation or down-regulation of goat *PRLR* long form causes the change of expression of different genes involved in fatty acids synthesis, and finally controls the content of lipid droplets in culture of goat mammary epithelial cells. In contrast, none of these roles have been ascribed to the *PRLR* short form (Shi *et al*., 2016).

Conclusions

In this study, we provided the first contribution to the characterization of the genomic sequence of the prolactin receptor encoding gene (*PRLR*) in the Mediterranean river buffalo. Based on the detected high degree of genetic variability, we defined at least 6 alleles at this *locus* each codifying for a different predicted amino acid sequences, four of which specific of the Mediterranean breed (Figure

3). All the detected mutations represent the first examples of markers found at the *PRLR* in this breed. The deletion of a CACTACC heptamer at exon 10 (3'UTR) can be considered as a good molecular markers to distinguish between the milk of different origins (e.g. from Mediterranean buffalo *vs* all others mammalian) This finding would be useful for the dairy industry. In fact, the buffalo dairy sector is one of the most affected by frauds. One of the main goal of the Consortium for the "Mozzarella di bufala Campana" PDO (Protected Designation of Origin) is to prevent the deception of consumers with similar cheese products made of less-expensive ingredients (milk of other species, curd imported from other countries, etc.). Conversely, the authentic buffalo mozzarella must be prepared only with fresh milk of Italian Mediterranean breed reared in specific geographical areas of Southern Italy, and following well definite guidelines. Finally, our data revealed that SNPs detected in *PRLR* gene could influence OBCFA content and, consequently, milk fat composition. Therefore, the use of these polymorphisms can be a useful tool for the improvement of buffalo milk quality by marker assisted selection.

Conflict of interest

The Authors have no conflict of interest

Acknowledgments

This work was financially supported by the Italian Ministry for Agriculture and Forestry Policy – MiPAAF (INNOVAGEN project)

References

Azevedo L., Serrano C., Amorim A. & Cooper D.N. (2015) Trans-species polymorphism in humans and the great apes is generally maintained by balancing selection that modulates the host immune response. *Human Genomics* **4**, 9–21.

Bignon C., Binart N., Ormandy C., Schuler L.A., Kelly P.A. & Djiane J. (1997) Long and short forms of the ovine prolactin receptor: cDNA cloning and genomic analysis reveal that the two forms arise by different alternative splicing mechanisms in ruminants and in rodents. *Journal of Molecular Endocrinology* **19**, 109–20.

Bole-Feysot C., Goffin V., Edery M., Binart N. & Kelly P.A. (1998) Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockoutmice. *Endocrine Reviews* **19**, 225–68

Buck K., Vanek M., Groner B. & Ball R.K. (1992) Multiple forms of prolactin receptor messenger ribonucleic acid are specifically expressed and regulated in murine tissues and the mammary cell line HC11. *Endocrinology* **130**, 1108–14.

Chu M.X., Mu Y.L., Fang L., Ye S.C. & Sun S.H. (2007). Prolactin receptor as a candidate gene for prolificacy of small tail Han sheep. *Animal Biotechnology* **18**, 65–73.

Clarke D.L., Arey B.J. & Linzer D.I.H. (1993) Prolactin receptor messenger ribonucleic acid expression in the ovary during rat estrous cycle. *Endocrinology* **133**, 2594–603.

Clarke D.L. & Linzer D.I.H. (1993) Changes in prolactin receptor expression during pregnancy in the mouse ovary. *Endocrinology* **133**, 224–32.

Cosenza G., Ianaccone M., Pico B.A., Gallo D., Capparelli R. & Pauciullo A. (2017a) Molecular characterization, genetic variability and detection of a functional polymorphism influencing the promoter activity of OXT gene in goat and sheep. *Journal of Dairy Research* **84(2)**, 165-9.

Cosenza G., Macciotta N.P.P., Nudda A., Coletta A., Ramunno L. & Pauciullo A. (2017b) A novel polymorphism in the Oxytocin receptor encoding gene (OXTR) affects milk fatty acid composition in Italian Mediterranean river buffalo. *Journal of Dairy Research* **84(2)**, 170-80.

Gabriel S.B., Schaffner S.F., Nguyen H., Moore J.M., Roy J., Blumenstiel B., Higgins, J., DeFelice M., Lochner A., Faggart M., Liu-Cordero S.N., Rotimi C., Adeyemo A., Cooper R., Ward R., Lander E.S., Daly M.J. & Altshuler D. (2002) The structure of haplotype blocks in the human genome. *Science* **296**, 2225–9.

Goossens M. & Kan Y.W. (1981) DNA analysis in the diagnosis of hemoglobin disorders. *Methods in Enzymology* **76**, 805–17.

Hayes H., Le C.C. Goubin G., Mercier D., Payen E.J., Bignon C. & Kohno K. (1996) Localization of ZNF164, ZNF146, GGTA1, SOX2, PRLR and EEF2 on homeologous cattle, sheep, and goat chromosomes by fluorescent in situ hybridization and comparison with human gene map. *Cytogenetics and Cell Genetics* **72**, 342–6.

Hedrick P.W. (2006) Genetic polymorphism in heterogeneous environments: the age of genomics. *Annual Review of Ecology, Evolution, and Systematics* **37**, 67–93.

399 Hou J.X., An X.P., Song Y.X., Wang J.G., Ma T., Han P., Fang F. & Cao B.Y. (2013) Combined
400 effects of four SNPs within goat PRLR gene on milk production traits. *Gene* **529**, 276–81.
401
402 Hou J.X., Fang F., An X.P. Yan Y., Ma T., Han P., Meng F.X., Song Y.X., Wang J.G., Cao B.Y.
403 (2014) Polymorphisms of PRLR and FOLR1 genes and association with milk production traits in
404 goats. *Genetics and Molecular Research* **13(2)**, 2555–62.
405
406 Hou J.X., An X.P., Han P., Peng J.Y. & Cao B.Y. (2015) Two missense mutations in exon 9 of caprine
407 PRLR gene were associated with litter size. *Animal Genenetics* **46(1)**, 87–90.
408
409 Iso-Touru T., Kantanen J., Li M.H. Gizejewski, Z. & Vilkki J. (2009) Divergent evolution in the
410 cytoplasmic domains of PRLR and GHR genes in Artiodactyla. *BMC Evolutionary Biology* **9**, 172.
411
412 Javed R., Gautam S.K., Vijh R.K. & Tantia M.S. (2011a) Six novel PCR-RFLP loci in milk quality
413 candidate genes in Bubalus bubalis. *International Journal of Livestock Production* **2(6)**, 79–83.
414
415 Javed R., Gautam S.K., Vijh R.K. & Tantia M.S. (2011b) Characterization of PRLR and PPARGC1A
416 genes in buffalo (Bubalus bubalis). *Genetics and Molecular Biology* **34**, 592–4.
417
418 Kaneda T. (1991) Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic
419 significance. *Microbiological Reviews* **55**, 288–302.
420
421 Klein J. (1987) Origin of major histocompatibility complex polymorphism: the trans-species
422 hypothesis. *Human Immunology* **19**, 155–62.
423

Lenstra J.A., Van Boxtel J.A.F., Zwaagstra K.A. & Schwerin M. (1993) Short interspersed nuclear elements (SINE) sequences of the Bovidae. *Animal Genetics* **24**, 33–9.

Lewis B.P., Burge C.B. & Bartel D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120(1)**, 15–20.

Lü A., Hu X., Chen H., Dong Y., Zhang Y. & Wang X. 2011. Novel SNPs of the Bovine PRLR Gene Associated with Milk Production Traits. *Biochemical Genetics* **49**, 177–89.

Mackie R.I., White B.A. & Bryant M.P. (1991) Lipid metabolism in anaerobic ecosystems. *Critical Reviews in Microbiology* **17**, 449–79.

Pauciullo A., Shuiep E., Cosenza G., Ramunno L. & Erhardt G. (2013) Molecular characterization and genetic variability at κ-casein gene 1 (CSN3) in camels. *Gene* **513**, 22–30.

Pauciullo A., Giambra I.J., Iannuzzi L. & Erhardt G. (2014) The β-casein in camels: molecular characterization of the CSN2 gene, promoter analysis and genetic variability. *Gene* **547**, 159–68.

Ramunno L., Cosenza G., Rando A, Illario R., Gallo D., Di Berardino D. & Masina P. (2004) The goat αs1-casein gene: gene structure and promoter analysis. *Gene* **334**, 105–11.

Shi D.S., Wang J., Yang Y., Lu F.H., Li X.P. & Liu Q.Y. (2012) DGAT1, GH, GHR, PRL and PRLR polymorphism in water buffalo (Bubalus bubalis). *Reproduction in Domestic Animals* **47**, 328–34.

Shi H., Zhang T., Yi, Y., Wang H. & Luo J. (2016) Long form PRLR (lPRLR) regulates genes involved in the triacylglycerolsynthesis in goat mammary gland epithelial cells. *Small Ruminant Research* **139**, 7–14.

Skrzypczak E., Babicz M. & Pastwa M. (2015) Effect of Prolactin Receptor (PRLR) and Beta-Casein (CSN2). Gene Polymorphism on the Chemical Composition of Milk Sows. *Folia Biologica (Kraków)* **63(2)**, 135–44.

Stefanov I., Baeten V., Abbas O., Colman E., Vlaeminck B., De Baets B. & Fievez V. (2010) Analysis of milk odd- and branched-chain fatty acids using Fourier transform (FT)-Raman spectroscopy. *Journal of Agricultural and Food Chemistry* **58**, 10804–11.

Tomás A., Casellas J., Ramírez O., Muñoz G., Noguera J.L. &, Sánchez A. (2006) High amino acid variation in the intracellular domain of the pig prolactin receptor (PRLR) and its relation to ovulation rate and piglet survival traits. *Journal of Animal Science* **84(8)**, 1991–8.

Trott J.F., Hovey R.C., Koduri S. & Vonderhaar B.K. (2003) Alternative splicing to exon 11 of human prolactin receptor gene results in multiple isoforms including a secreted prolactin-binding protein. *Journal of Molecular Endocrinology* **30**, 31–47.

Wang L.P., Geng R.Q., Zhang X.N. & Sun W. (2015) Identification of SNPs within the PRLR gene and effects on maternal behavior in sheep. *Genetics and Molecular Research* **14(4)**, 17536–43.

Zhang J.L., Zan L.S., Fang P., Zhang F., Shen G. & Tian W.Q. (2008) Genetic variation of *PRLR* gene and association with milk performance traits in dairy cattle. *Canadian Journal of Animal Science* **88**, 33–9.

Zi X.D., Chen D.W. & Wang H.M. (2012) Molecular characterization, mRNA expression of prolactin receptor (PRLR) gene during pregnancy, nonpregnancy in the yak (Bos grunniens). *General and Comparative Endocrinology* **175**, 384–8.

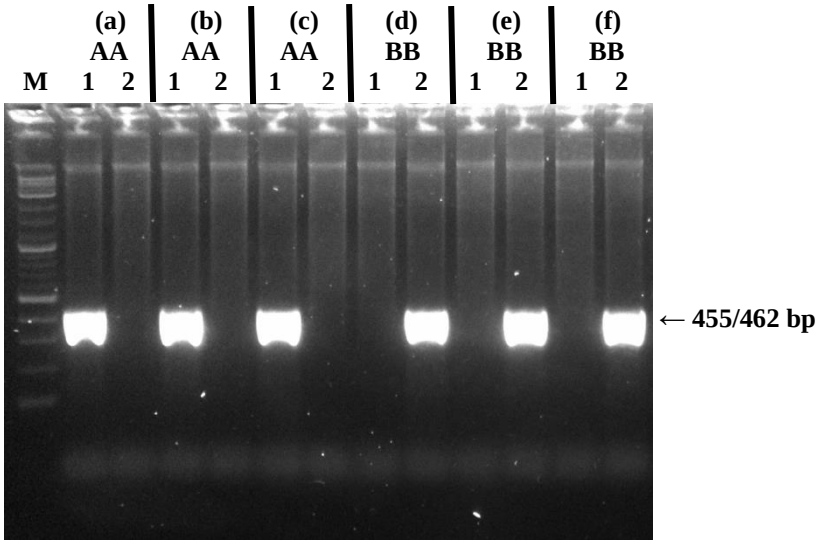
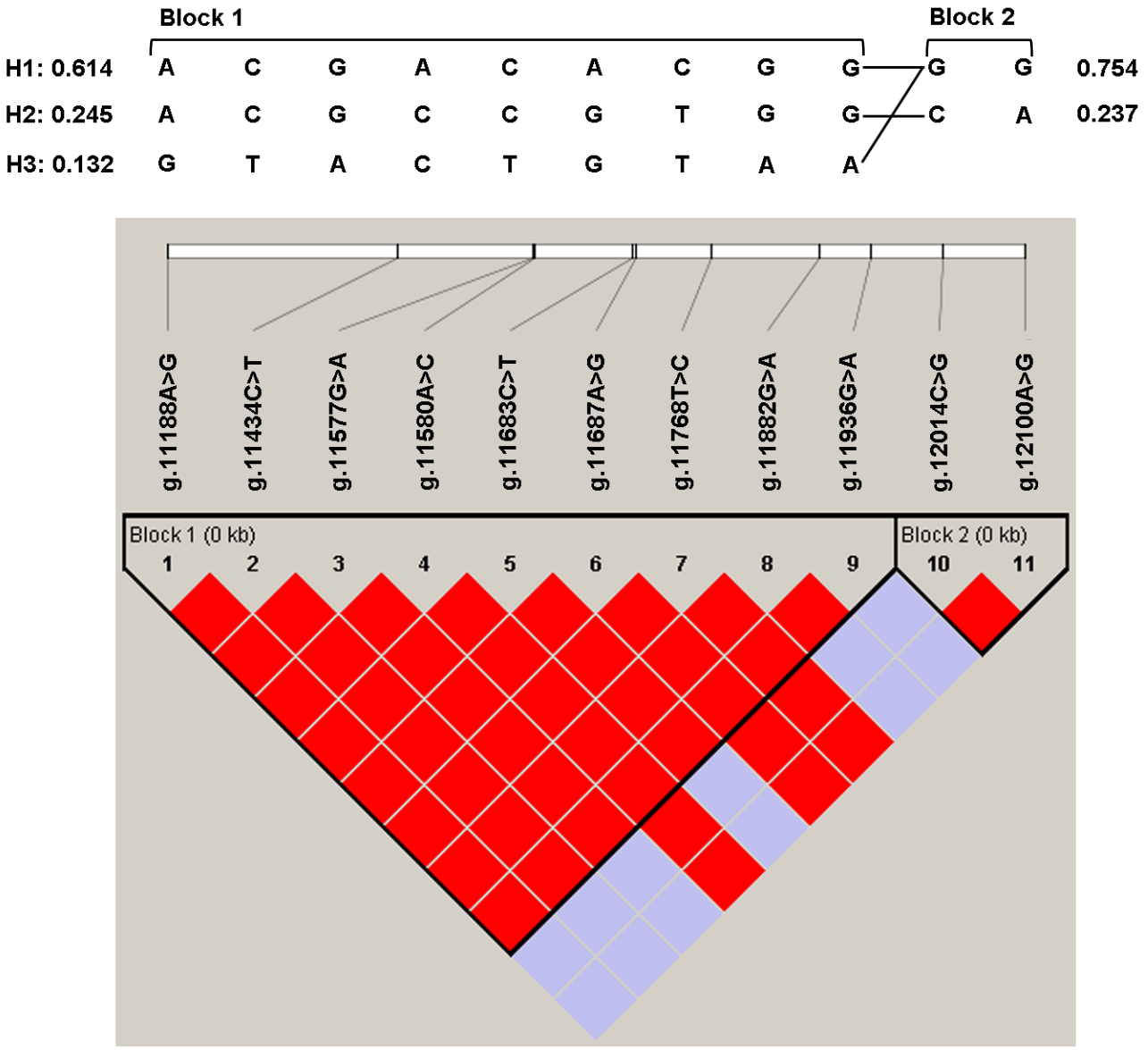


Fig. 1 Identification by AS-PCR of carriers of the heptamer CACTACC at the exon 10 of the *PRLR* gene in Mediterranean (a), Murrah (b) and Mehsana (c) river buffaloes, goat (d), sheep (e) and cattle (f).
AA, homozygous for the "non CACTACC insertion"; BB, homozygous for the "CACTACC insertion";
1, "non CACTACC insertion" primer; 2, "CACTACC insertion" primer, M, Marker (2-Long DNA Ladder (0.1-10 kb) (Biolabs).

Fig. 2. Linkage disequilibrium analysis, observed haplotypes (H1, H2 and H3) and their relative frequencies at the Mediterranean river buffalo *PRLR locus* (exon 10).



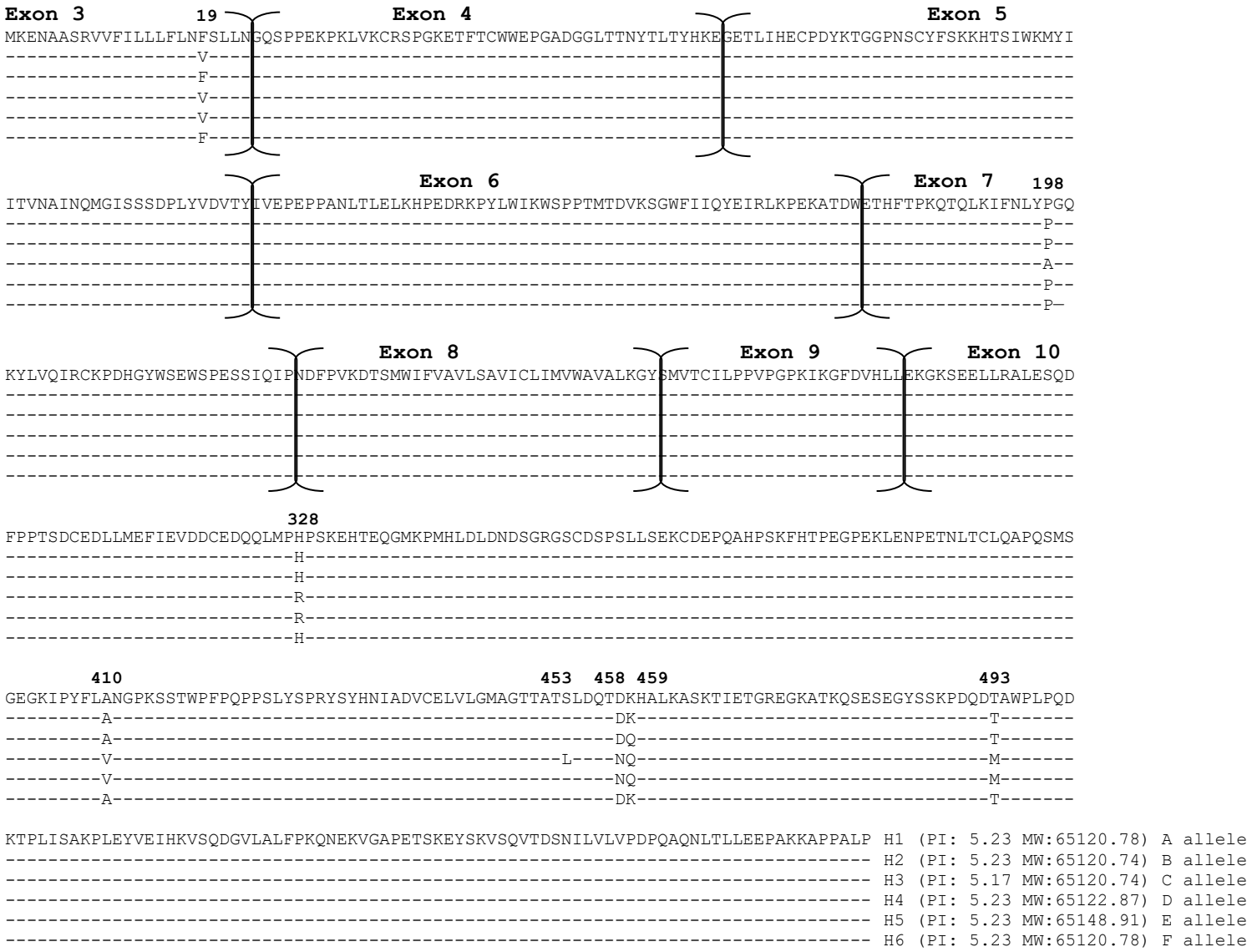


Fig. 3. Amino acid comparison (deduced mRNA) of different haplotypes (H1 to H6) observed at the buffalo *PRLR locus*. Dashes represent nt identical to those in upper lines. PI: Isoelectric Point; MW: Molecular Weight.

Description	Mutation	aa change
Exon 3	g.114T>G	p.Phe19Val
Intron 3	g.216T>C	
	g.689A>G	
	g.947A>G	
	g.1046C>A	
	g.1119_1121insA	
	g.1999G>A	
	g.2100A>G	
	g.2550T>G	
Intron 4	g.3266C>T	
	g.3720G>A	
	g.3777C>T	
	g.3835G>A	
Intron 6	g.7011G>T	
	g.7333C>T	
Exon 7	g.7401C>G	p.Pro198Ala
Intron 7	g.7953T>A	
	g.8948_8951delG	
Intron 8	g.9504G>T	
Intron 9	g.10240G>A	
	g.10254C>T	
	g.10276C>T	
	g.10352A>T	
	g.10494C>T	
	g.10558_10561delAGTC	
	g.11007C>T	
Exon 10	g.11188A>G	p.His328Arg
	g.11434C>T	p.Ala410Val
	g.11577G>A	p.Asp458Asn
	g.11580A>C	p.Lys459Gln
	g.11683C>T	p.Thr493Met
	g.11687A>G	p.Ala494
	g.11768T>C	p.Asp521
	g.11882G>A	p.Pro559
	g.11936G>A	p.Pro577
Exon 10 3'UT	g.12014C>G	
	g.12100A>G	

Tab. 1. Polymorphisms detected at Mediterranean river buffalo *PRLR locus*

Tab. 2. Genotyping data, allele frequency, relative frequencies of buffalo cows of the SNP g.11188A>G at the exon 10 of the *PRLR* gene in Mediterranean river buffalo population.

Position	**Genotype distribution**					**Allele freq**		**Relative frequencies used in the model**		
		AA	AG	GG	TOT	A	G	**Genotype**	**n. of cows**	**n. of farms**
g.11188A>G	Obs.	253	49	6	308	0.9	0.1	AA	82.14	14
	Exp.	250.02	54.96	3.02				AG	15.91	
	1 degree of freedom P value <0.05; χ^2= 3.84							GG	1.95	
								Total	100	