Sequencing of lipoprotein lipase gene in the Mediterranean river buffalo identified novel variants affecting gene expression

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3   The *LPL* gene plays an essential role in the lipid metabolism being responsible for the

4   composition of fat in adipose tissue as well as in milk. It has been poorly investigated in farm animals

5   and little information was reported for the buffalo. Among ruminants, the buffaloes produce milk with

6   a higher fat content; therefore, the improvement of this trait is one of the main goals for the progress

7   of the species. We identified novel functional polymorphisms, using a combined approach based on

8   sequencing, dual colour electrophoretic mobility shift assay and q-PCR. Our data revealed that the

9   SNP g.-446A>G in the promoter affects the binding of a Sp1 transcription factor, resulting in the

10   overexpression of the gene with with potential effects on milk quali-quantitative production.

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Sequencing of Lipoprotein lipase (*LPL*) gene in the Mediterranean river buffalo (*Bubalus bubalis*) identified novel variants affecting gene expression

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NOVEL VARIANTS AT BUFFALO *LPL* AFFECT GENE EXPRESSION

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ABSTRACT

Lipoprotein lipase is a key enzyme for the lipid metabolism playing a fundamental role in the composition of fat in adipose tissue as well as in milk. Seldom Lipoprotein lipase (*LPL)* gene was investigated in dairy ruminants, and it has been barely studied in river buffalo so far. The aim of this work was to explore the genetic diversity of *LPL* and its promoter and to identify functional mutations using a combined approach based on sequencing, dual colour electrophoretic mobility shift assay (EMSA) and quantitative PCR (q-PCR). Thirteen consensus sequences for transcription factors were found in the promoter. A total of 11 SNPs were detected and the attention was focused on the SNPs with potential functional effects: g.-466A>G, because the presence of G created a consensus motif for the transcription factor Sp1, and g.107A>G, which was the only exonic SNP. PCR-RFLP methods for genotyping were developed for the two SNPs and the allele frequencies were calculated. A strong linkage disequilibrium (D' = 1; r^2 = 0.903) was found between the two SNPs. The dual colour EMSA demonstrated that only the genotype g.-446GG induced the binding of the Sp1 transcription factor, resulting in the overexpression of the gene (~2.5 fold), as confirmed by the q-PCR results. This study adds further knowledge on the structure of the *LPL* gene and its expression in river buffalo, with potential effects on milk quali-quantitative production.

Keywords: Mediterranean river buffalo, *LPL* gene, promoter, Gene expression, Sp1 transcription factor.

INTRODUCTION

Milk fat is mostly composed of triglycerides (over 95%), whose fatty acids (FAs) can be originated by ruminal fermentation of carbohydrates (*de novo* synthesis) or come directly from the plasma by the action of lipoprotein lipase (LPL) (Barber *et al.*, 1997).

Lipoprotein lipase is the key enzyme involved in metabolism, transport and regulation of lipid serum levels. It acts on the surface of the capillary endothelium by hydrolysing triglycerides contained in circulating chylomicrons and very low density lipoprotein (VLDL) to provide free FAs to tissues. Regulation of Lipoprotein lipase (*LPL*) gene is very complex and responds to dietary and hormonal changes and environmental conditions being tissue-specific. The major tissues thought to control the circulating levels of plasma lipoproteins are the adipose and muscular tissues. However, *LPL* is expressed in other sites, including the mammary gland and other body compartments such as the nervous system, heart, liver, pancreatic islet cells, lungs, etc. (Merkel *et al.*, 2002).

The *LPL* gene has been deeply investigated in several species and single nucleotide polymorphisms have been reported in cattle (Lien *et al.*, 1995; Oh *et al.*, 2013; Tank & Pomp, 1994) and in yak (Ding *et al.*, 2012), where *LPL* has been proposed as major candidate gene for lipid deposition in the carcass and fatty acid composition. Marchitelli *et al.* (2013) reported a significant effect of the *LPL* g.25732A allele on milk fat percentage in the Piedmontese cattle breed.

In goats, the sequencing of the *LPL* cDNA allowed the identification of a missense mutation associated with milk fat content (Badaoui *et al.*, 2007). In the same species, Crepaldi *et al.* (2013) found an association between the transversion C50G at the exon 1 and milk yield, thus reporting for the first time an effect of *LPL* gene on this trait.

Among the farm animals, river buffalo is the species producing the highest milk fat, with an average content estimated in about 8.0% (http://www.anasb.it/home.htm). Milk yield and composition are of great economic importance for the dairy industry in Italy where this species is mainly reared for the production of *mozzarella* cheese PDO (Protected Denomination of Origin – Reg. EC 510/2006). The increasing demand of this product both on the national and international

84 market facilitated the development of the whole buffalo dairy industry, which currently involves 102

85 cheese factories and more than 20,000 operators, with a consolidated turnover estimated in about 720

86 million of euros (ISMEA, 2016). In this context, the discovery of genetic markers affecting milk yield

87 and composition could contribute to accelerate the genetic improvement of the species.

88 Therefore, the aim of this study was to explore the genetic diversity of *LPL* gene in the Italian

89 Mediterranean breed, to describe the main regulatory elements of the promoter and to investigate

90 possible effects of the polymorphism on the gene expression.

91

92 **MATERIAL AND METHODS**

93 *Sample collection and nucleic acid isolation*

94 A total of 523 river buffalo cows reared in 14 farms located in Campania region (Southern

95 Italy) were considered in this study. From all cows blood samples were collected in collaboration

96 with the Italian National Association of Buffalo Breeders (ANASB) during the routine prophylaxis

97 of each farm. For this reason, the Animal Care and Use Committee approval was not necessary. In

98 addition, six individual milk samples were collected after genotyping at the SNPs g.107A>G and g.-

99 446A>G for the isolation of RNA in order to perform the qPCR.

100 Genomic DNA was isolated using the NucleoSpin Tissue kit (Macherey-Nagel, Düren,

101 Germany). Total RNA was isolated from milk somatic cells by Tri-Reagent® (Sigma-Aldrich, Milano,

102 Italy) following the manufacturer's guidelines. Traces of contaminating DNA were removed using

103 DNase I treatment (Thermo Fisher Scientific).

104 DNA and RNA concentrations and OD ratios of the samples were measured with the

105 Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

106

107 *Sequencing and bioinformatics*

108   Genomic DNA of 14 individual samples randomly chosen was amplified by iCycler (BioRad)

109 using primers designed on the buffalo genome sequence (EMBL acc. no. AWWX01438720.1)

110 (Supplementary table 1).

111   The PCR reaction mix (25 µl) comprised: 50 ng of genomic DNA, 1X PCR Buffer (Promega,

112 Madison, WI, USA), 2.5 mM $MgCl_2$, 5 pmol of each primer, dNTPs 200 µM each, 1 U of *Taq* DNA

113 Polymerase (Promega). The thermal conditions were: 95°C for 4 min, 35 cycles at 95°C for 45 s,

114 annealing for 45 s, at temperatures depending on the amplicon (Supplementary table 1), and extension

115 at 72°C for 45 s. A final extension was carried out at 72°C for 5 min.

116   Product specificity was confirmed by electrophoresis in 1.5% agarose gel in 0.5X TBE buffer,

117 stained with Sybr Green. PCR products were purified and sequenced on both strands at Microsynth

118 (Vienna, Austria).

119   Homology search, comparison among sequences, and multiple alignment for SNP discovery

120 were accomplished using DNAsis-MAX (Hitachi). The putative binding sites for transcription factor

121 at the promoter were searched by Transfact 7.0 database under the most stringent condition of analysis

122 (85% as minimum binding score and 100% similarity of the sequence to consensus matrix).

123

124 ***Genotyping of the SNPs g.107A>G and g.-446A>G***

125   The entire panel of 523 animals was genotyped in out-sourcing (http://kbioscience.co.uk)

126 using a competitive allele specific PCR system (KASPar) for the SNP g.107A>G. Also, a PCR-

127 RFLP method was set up for the same SNP to facilitate future home made genotyping. In brief, 10µl

128 of exon 1 amplicons from informative samples were digested with 1µl of Fastdigest *Dde* I (5'-

129 C↓TNAG-3') endonuclease (Thermo Fisher Scientific Inc.) for 10 min at 37°C according to

130 manufacturer's guidelines.

131   Forty samples, randomly chosen from the panel of 523 buffaloes, were genotyped also for

132   the SNP g.-446A>G located in the promoter by digesting the amplicon with the endonuclease *Ssi* I

133   (5'-C↓CGC-3') overnight at 37°C in the same conditions described above.

134   The digestion products were resolved on 2.5% agarose gel in 1X TBE buffer, stained with

135   Sybr Green.

136

Dual colour Electrophoretic Mobility Shift Assay (EMSA)

138   Single stranded DNA probes (table 1) corresponding to the nucleotides -458 to -434 of the

139   *LPL* promoter were co-denatured at 95°C for 5 min in 0.2X SSC buffer and annealed at room

140   temperature for 2 hours with their complementary sequence to obtain double strand probes

141   (homozygous AA and GG). Concentrations and OD ratios were measured with the Nanodrop ND-

142   1000 spectrophotometer as reported previously and dilutions were prepared to a final concentration

143   of 80ng/µl.

144   Electrophoretic Mobility Shift Assay reactions were carried out according to Gu et al. (2019).

145   Briefly, binding reactions were set-up by incubating 0.5 BFU of Sp1 transcription factor (Enzo Life

146   Science Inc., Farmingdale, NY, USA) in 1X Enzo buffer (EB) with 40 ng DNA probe. After

147   incubation at 37°C for 20 min, the samples were loaded in 5% native polyacrylamide gel and run at

148   150V for 90 min in pre-chilled TBE 0.5X according to (Jing et al., 2003). Staining was performed as

149   sequential steps in dual colour, Sybr™ Green for the DNA and Sypro™ Ruby for the protein,

150   according to the manufacturer's guidelines of EMSA kit (ThermoFisher Scientific). The image was

151   acquired using the Gel Doc™ EZ System (Bio-Rad) and the digital images pseudocolored and

152   overlaid.

153

Quantitative real-time PCR analysis (qPCR)

155   Comparative *LPL* gene expression was examined in 6 Mediterranean river buffaloes chosen

156   according to the genotypes at the SNPs g.-466A>G and g.107A>G (2 samples double homozygous

AA, 2 samples double heterozygous AG and 2 samples double homozygous GG). The animals

belonged to the same farm, and were at the 3rd lactation, at 210 ± 11 days in milk, free from mastitis.

Reverse transcription was accomplished by Verso cDNA synthesis kit (ThermoFisher

Scientific) starting from 100 ng of total RNA in a final reaction volume of 20µl and using a mixture

of oligo dT_{18} and Random Hexamer as primers, according to the protocol suggested by the company.

Quantitative PCR was performed using 2X QuantiTect SYBR Green PCR Master Mix in

presence of ROX passive reference dye (Qiagen). Analysis was carried out with a Mini Opticon Real-

time PCR System (Bio-Rad) for 40 cycles at 95°C for 15 s, 55°C for 15 s and 72°C for 20 s.

Amplification specificity was checked using melting curve following the manufacturer's instructions.

Primers used for *LPL, β-actin* and 18S rRNA expression are listed in table 1. Two biological replicates

per sample were analysed and each replicate was amplified in triplicate (technical replicates). Relative

gene expression of *LPL* was determined using *β-actin* and 18S rRNA as endogenous controls using

the geometric mean values for normalization. Results are reported as fold-change relative to the mean.

Statistical analysis

Allelic frequencies were calculated by Popgene software (University of Alberta, Edmonton,

Alberta, Canada). Haploview 4.2 was used to estimate the rate of linkage disequilibrium (D' and r^2)

between the SNPs g.107A>G and g.-446A>G. Unpaired Students's t-test was applied to evaluate the

differences in the gene expression.

RESULTS AND DISCUSSION

The DNA regions covering the promoter, the 5'UTR and all coding regions until the stop

codon of the river buffalo *LPL* gene were amplified and sequenced, for a total of 5510 bp.

The analysis of the first 750 bp of the river buffalo *LPL* promoter greatly contributed to

evaluating the factors involved in the regulation of the gene expression. In this respect, *LPL* was

scarcely investigated in ruminants, with information limited to a short report in sheep (Volpe *et al.*, 1994).

The bioinformatics analysis revealed thirteen consensus sequences for transcription factors (figure 1). Twelve of them were equally represented: three TATA-boxes (-301/-306; -606/-611; -648/-653), three Oct-1 (-66/-75; -155/ -164; -559/-608), three Sp1 (-3/+7; -6/-15; -444/-449) and three C/EBPα (-57/-64; -89/-95; -545/-551). In addition, a motif AP2 was found in position +34/+41.

Part of these regulatory elements have been described also in human where transfection experiments in adipocytes identified proximal positive (-368 to -35) and distal negative (-724 to -565) *cis*-acting motifs (Previato *et al.*, 1991). In particular, the proximal regulatory region was shown to contain multiple conserved binding sites including C/EBP and Oct-1.

C/EBPs are known to be crucial components of terminal adipocyte development and play, together with PPARγ, a master role in the regulation of adipogenesis in the network of transcription factors which interact at different stages of the process (Farmer, 2006). *LPL* is one of the adipocyte-specific genes that are trans-activated by C/EBPα (Olofsson *et al.*, 2008). In river buffalo, C/EBPα motifs have been amply found also in the promoter region of *SCD*, another gene involved in lipid metabolism, and associated with milk fat quality (Pauciullo *et al.*, 2012b).

Oct-1 belongs to octamer-binding proteins (OCTs), a group of highly conserved transcription factors that specifically bind to the octamer motif (ATGCAAAT) and to closely related sequences found in promoters and enhancers (Zhao, 2013). Oct-1 regulates the expression of a variety of genes, including κ- (Pauciullo *et al.*, 2013) and β-casein genes (Pauciullo *et al.*, 2014), oxytocin (Cosenza *et al.*, 2017a) and immunoglobulin genes (Dreyfus *et al.*, 1987). Furthermore, Oct-1 it is not a strong transcriptional activator by itself, but in conjunction with other co-activators as TATA-binding proteins (Zwilling *et al.*, 1994).

In human, Oct-1 plays a major role in the modulation of *LPL* gene expression with three elements involved, one distal and two proximal (Previato *et al.*, 1991). In fact, the deletion of the region containing the distal octamer sequence resulted in a 75-100% increase of activity. Conversely,

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the deletion of the region with the two proximal octamers resulted in the decrease of transcription

activity of approximately 65% (Previato *et al.*, 1991). Human octamer motifs are conserved in mouse

and chicken *LPL* promoters (Yang *et al.*, 1995) and homology analysis with buffalo promoter

confirmed a similarity of 96%. Therefore, it is reasonable to suggest that the presence of conserved

Oct-1 motifs leads to similar regulation mechanisms of *LPL* gene expression mediated by this

transcription factor.

Two cis-acting regions of the human *LPL* promoter, known as LP-α

(GCGACTATCTTCTTTCACTTATCATAACTCAATACGG) and LP-β

(GGACGCAATGTGTGTCCCTCTATCCCTACATTGACTTTGC), were also considered important

for transcriptional activation of the gene during the adipocyte differentiation (Enerbäck *et al.*, 1992).

The comparative analysis of the LP-α and LP-β consensus sequences with the reference representative

genomes available and annotated for the *LPL* showed that the human LP-α sequence was found across

many species (with different level of homology), including river buffalo with 85% of similarity

(Figure 1). Conversely, no significant similarity was found in buffalo for the LP-β, which is present

only in primates, in the sea otter, and in the Hawaiian monk seal (similarity ranging from 85 to 100%).

This result suggests a major impact of LP-α on the *LPL* regulation among different species. Further

studies will be necessary to evaluate the real influence of this element on the transcriptional regulation

of *LPL* in river buffalo.

The sequence analysis revealed 11 SNPs in total (Table 2). Although the sequenced region

covers only 20.3% of the *LPL* (taking as reference the bovine gene sequence NC_037335.1 and the

buffalo genome AWWX01438720.1), the variability observed in river buffalo is high compared to

that reported for other ruminants (Badaoui *et al.*, 2007; Crepaldi *et al.*, 2013; Oh *et al.*, 2013). This

finding is probably the result of the lower selective pressure applied on buffalo compared to other

ruminants. Therefore, the natural genetic diversity is more preserved in this species, as happens also

in humans, where about 100 polymorphisms have been identified in the *LPL* gene, mostly associated

with the occurrence of familial hyper-lipidemia and atherosclerosis due to a reduction or loss of LPL function (Merkel *et al.*, 2002).

Six polymorphic sites were detected in the intron 3. None of them affected key sites of the spliceosome machinery (acceptor sites, branch points, poly-pyrimidine tracts, donor sites). Therefore, these mutations are not expected to influence the gene expression.

The other five SNPs were observed in the promoter (four SNPs) and in exon 1 (one SNP). Of these, two are of special interest for their potential functional effects. The mutation g.-446A>G falls within the consensus sequence of a Sp1 transcription factor, which also regulates the expression of other lipogenic genes in river buffalo as, for instance, the stearoyl CoA desaturase gene (*SCD*), as recently demonstrated by Gu *et al*. (2019). Conversely, the exonic mutation (g.107G>A) falls in the 5' un-translated region (UTR), which is a non-coding region, but plays an important role in the translational regulation. In fact, in addition to consensus sequences for transcription factors, it contains Kozak consensus sequences and cis-acting regulatory elements known as upstream Open Reading Frames (uORFs) (Wethmar *et al.*, 2010).

The PCR-RFLP protocols set up for the SNP genotyping proved to be effective for the allele discrimination: for g.107G>A the digestion of the PCR product (200 bp) with the *Dde*I endonuclease produced two fragments (195 and 5 bp) for the G allele, and three fragments (103, 92, 5 bp) for the A allele (Figure 2A). For the SNP g.-446A>G, using the endonuclease *Ss*I, the allele A remains undigested (151 bp), whereas the allele G is restricted into two fragments (84 and 67 bp) (Figure 2B).

Frequencies of 0.63 and 0.68 were calculated for the alleles g.107G and g.-446G respectively. At both SNPs the genotype frequencies were in agreement with the Hardy-Weinberg equilibrium.

A strong linkage disequilibrium (D'=1) was found between these two SNPs and the high level of r^2 (0.903) allows the imputation of an allele at one SNP, known the allele at the other SNP.

In order to investigate the effects of the SNP g.-446A>G on the *LPL* expression, we tested *in vitro* whether a genotype-dependent binding reaction exists between the transcription factor Sp1 and this genetic marker, using the electrophoretic mobility shift assay (EMSA). This technique is a

powerful tool to assess the selective binding of a protein to a double stranded DNA. The specific protein-DNA interaction results in the reduced migration rate of the complex compared with the free DNA (Garner and Revzin, 1981). Therefore, dual colour EMSA experiments were performed using specific double stranded DNA constructs (green colour) corresponding to homozygous genotypes (g.-446AA and g.-446GG), each treated with a recombinant Sp1 transcription factor (red colour). The presence of the dual colour band (yellow) and the shift in the mobility of the DNA construct (Figure 3) demonstrated that the Sp1 interacted only with the genotype g.-446GG, generating a dual colour band (yellow) and a shift in the molecular weight of the DNA construct (Figure 3) confirming, *de facto*, the bioinformatics analysis (Figure 1). The transcription factors Sp1 are considered enhancer motifs for the basal expression of many genes, including *LPL*, whose transcription in human is stimulated by this transcription factor itself or by a synergistic interaction between Sp1 and SREBP-1 (Yang and Deeb, 1998).

The exclusive interaction of the transcription factor Sp1 with the promoter having genotype g.-446GG led us to investigate also the effect of the SNP on the *LPL* transcription level. The q-PCR showed a significant overexpression of the genotype GG (~2.5 fold higher) compared with both AA (P=0.0019) and AG (P=0.0017) genotypes (Figure 4), in agreement with the results of the EMSA experiments. In particular, the genotype GG induced the Sp1 binding, which consequently up-regulated the expression of the *LPL* gene.

These results could have practical implications for the improvement of milk quality. In fact, the up-regulation of the *LPL* in mammary gland is mediated by the anterior pituitary through the release of prolactin (Thomson, 1992), in agreement with hormonal regulation of the mammary gland during the lactation period and nutrient transport supporting the milk production (Bionaz and Loor, 2008). In addition, the change of *LPL* gene expression in milk somatic cells may affect the natural function of LPL enzyme, i.e. the hydrolysis of fatty acids from triglycerides and phospholipids in the milk.

284 LPL regulates the hydrolysis of circulating triglycerides carried by chylomicrons and VLDL

285 (Very Low Density Lipoprotein), and the uptake of fatty acids by most tissues, including mammary

286 gland and adipose tissues. In particular, lipoprotein lipase is critical for the uptake and secretion of

287 the long-chain fatty acids in milk (Iverson *et al.*, 1995). In the lactating females, LPL levels are almost

288 completely depressed in adipose tissue, while those in the mammary gland are high. As a

289 consequence, circulating fatty acids are directed to the mammary gland for milk fat production

290 (Iverson *et al.*, 1995). Therefore, LPL may affect the quali-quantitative characteristics of milk fat

291 through this mechanism. The effect of the *LPL* polymorphism on milk production in river buffalo is

292 under investigation.

293

294 **CONCLUSIONS**

295 The genetic progress of Italian river buffalo focuses on the improvement of the milk

296 production traits; therefore, the genes regulating fat metabolism are important targets of study. In the

297 present investigation, we provided the first description of *LPL* promoter and reported on the genetic

298 diversity found in that region and at exon level. The SNP g.-446A>G in the promoter region created

299 a consensus sequence for the transcription factor Sp1, whose binding activity was demonstrated to be

300 genotype-dependent. The specific bond to the GG genotype induced a significant increase in the *LPL*

301 gene expression, which could be associated with the milk yield and composition.

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415

1 **Table 1**. Oligonucleotide primers sequences, positions and applications.

Application	Gene	Region		Primer sequence	EMBL ID	Size (bp)	Endonuclease
Genotyping	*LPL*	Promoter	*Forward*	5'-GCAAGTTCAAGCTGCAGAAA-3'	AWWX01438720.1	151	*Ssi* I
			Reverse	5'-ATTTGGGCAATACCCAGCTT-3'			
		Exon 1	*Forward*	5'-GGAAACCTGCCGCTTCTA-3'		200	*Dde* I
			Reverse	5'-GCTCAGAGCCAGCAGAA-3'			
Realtime-PCR	*LPL*	Exon 1	*Forward*	5'-GGAAACCTGCCGCTTCTA-3'	AWWX01438720.1	200	-
			Reverse	5'-GCTCAGAGCCAGCAGAA-3'			
	β-actin	Exon 1	*Forward*	TCCTCCCTGGAGAAGAGCTA	NM_001290932	101	-
			Reverse	AGGAAGGAAGGCTGGAAGAG			
	18S rRNA	Exon 1	*Forward*	CGTTCTTAGTTGGTGG	NR_036642	76	-
			Reverse	GTAACTAGTTAGCATGC			
			Genotype	**Probes (5' to 3')**			
EMSA	*LPL*	promoter	AA	5'-AGGAGAATTGGC**A**GGGGAGGGGGGG-3'	AWWX01438720.1	25	-
			GG	5'-AGGAGAATTGGC**G**GGGGAGGGGGGG-3'			

2
3 Primers were designed by means of DNAsis software (Hitachi Software Engineering Co., San Bruno, CA).
4
5

1 **Table 2.**
2 Polymorphisms found at river buffalo *LPL*. European Variation Archive (EVA) BioProject Acc. No.
3 PRJEB28835
4

Location	Position (1)	Position (2)	Polymorphism
Promoter	-517	13606	A/T
	-446	13677	A/G
	-433	13690	A/G
	-358	13765	C/T
Exon 1	+107	14229	A/G
Intron 3	+33	27206	A/G
	+38	27211	A/G
	+43	27216	T/G
	+64	27237	A/G
	+70	27243	T/A
	+149	27322	A/G

5 (1) Numbering refers to each location and re-starts from +1 (exon 1 and intron 3) or -1 (promoter)
6 each time.
7 (2) Numbering refers to the buffalo genome sequence (EMBL ID: AWWX01438720.1)
8

```
                                                       LP-α
tatgcaaaggaaataataggatgtgcatcttctgtgactggcttcttttcacttagcataa   -699

                                                    TATA
caatacactttcaggctgtctggcctcaacattctgcctctgaggataaaatgttacttt    -639

                              TATA   Oct-1
gcatttcttgatgagtttgaggattgagtataatttgcataaggcaaaaattagaaacta    -579

                                  C/EBPα
gagcaagagggcttttctcaactacaagccaatccatattaccaaagttaaaacaccaga    -519

twgtgcaagttcaagctgcagaaaaatgtgagtccgtgggtccttagattgacattgtcc    -459

          Sp1
aggagaattggcrggggagggggggracaaaaaacaaaacagacactgcaagttacaaac    -399

aggagccaccaaaagaagctgggtattgcccaaatgtagtytaacaacttaacttccctt    -339

                              TATA
tcttagaaaacagatgattacagagtatttcaataaagccataaaaacctttgtttggt     -279

gtttgtgtaggggacccaggagagccagacctcttaggaaaagcaaattgccctcctaaa    -219

                                                          Oct-1
gaaagtaaaattttccttctgccctttccccttcttctccctggcagagttgaatctca     -159

ttacagtttgctcaaatatttgaaagtgaatttagtatcctccccccaacttaagatttt    -99

   C/EBPα                    Oct-1         C/EBPα
atagccaatagatgatgaggttcatttgcatattccaatcacataagcagccgtggagt     -39

                       Sp1           Sp1         5'UTR
ggaaacagtgtcggactcatttttctcctccccacctcctcctcdtccgaggaaacctgc    22

       AP2                 Exon 1
cgcttctagctccccaccctcccctttaaagggtgacttgctctgtgccagaccgctgct   82

ccagcctgctgccgccttgggctcrgcgggtctactgttctgtccgcgctcgcgcctggt   142

                                       M  E  S  K  A  L  L  L  L
gccctgcatctcctacagagggacatcccccgagATGGAGAGCAAGGCCCTACTTCTGCT   202

   A  L  S
GGCTCTGAGC...
```

Figure 1.

Key regulatory motifs found in the 5' promoter region of the river buffalo *LPL* gene. Numbering is relative to the first nucleotide of the first exon (+1). The 5' UTR (Un-Translated Region) of the exon 1 is reported in italics, whereas the coding region is in upper case. Genetic diversity is indicated in bold (w = a/t; r = a/g; y = c/t).
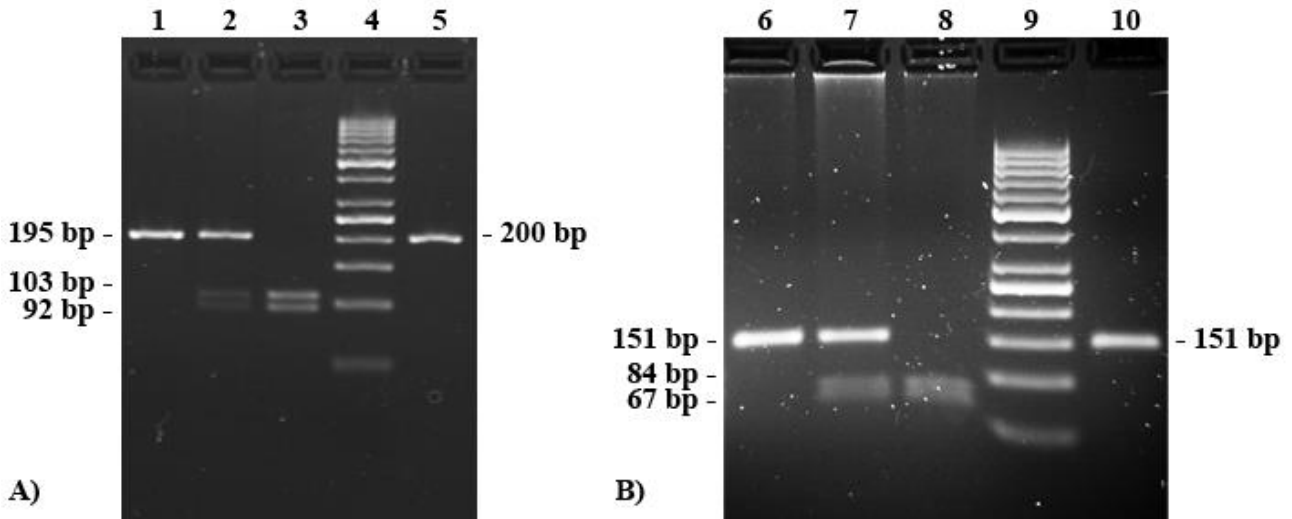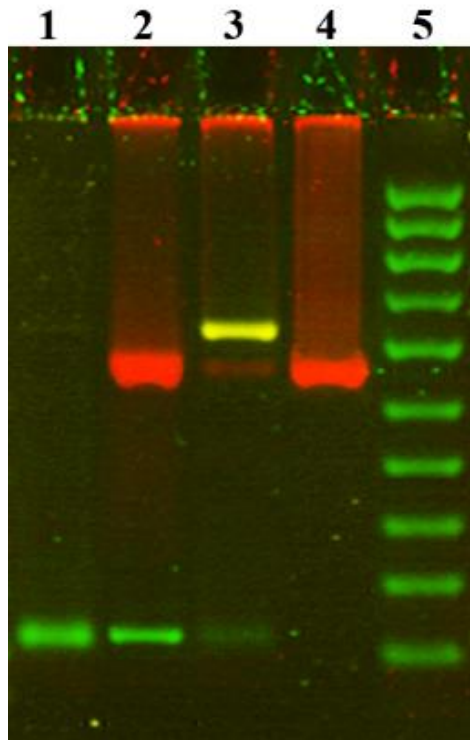
Figure 2.
A) Genotyping of the SNP g.107A>G in the exon 1 of river buffalo *LPL* by *Dde* I PCR-RFLP. Line 1, GG homozygous sample; line 3, AA homozygous sample; line 2, heterozygous sample. Line 4, GeneRuler 50 bp DNA Ladder (Thermo Scientific); line 5, undigested PCR product. The band 5bp long was not visible on the gel. **B)** Genotyping of the SNP g.-446A>G in the *LPL* promoter by *Ssi* I PCR-RFLP. Line 6, AA homozygous sample; line 8, GG homozygous sample; line 7, heterozygous sample. Line 9, GeneRuler 50 bp DNA Ladder (Thermo Scientific); line 10, undigested PCR product.
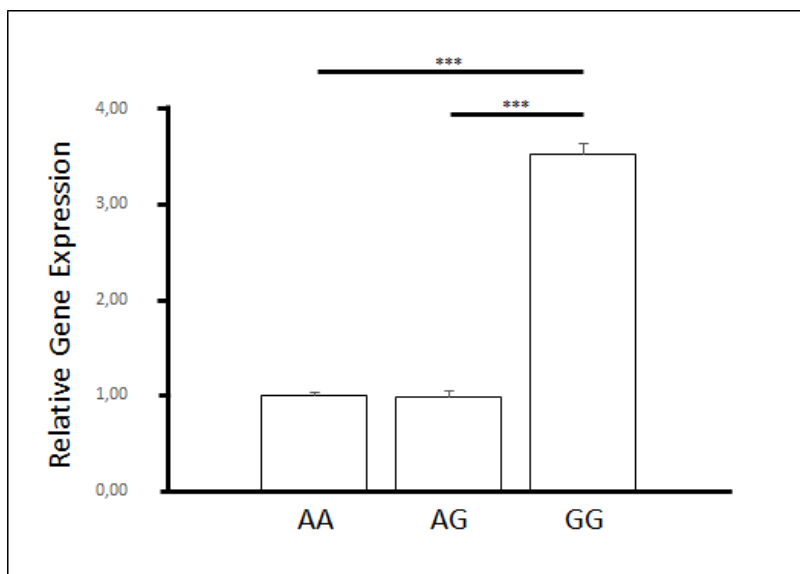
Figure 3
Dual colour electromobility shift assay (EMSA) testing the interaction between the *LPL* promoter and the transcription factor Sp1. Line 1, free DNA sample (Green); line 2, DNA probe with genotype AA (no binding); line 3, DNA probe with genotype GG (binding and shift in yellow); line 4, free Sp1 protein (Red); line 5, 20 bp DNA Ladder (Jena Bioscience).

Figure 4

Relative expression values (mean ± SEM) of LPL in 6 buffaloes (2 animals for each genotype at g.107A>G and g.-446A>G). Unpaired Student's *t*-test was used to assess the differences among the genotypes (***$P < 0.01$).

1

Supplementary table 1.

Primer sequences, position according to the genome sequence AWWX01438720.1, annealing temperature and amplicon size used for the sequencing of river buffalo *LPL* gene.

Region covered		Position	Primers	Sequence	Ta °C	Size (bp)
Promoter/Exon 1		13321-13340	*Forward*	5'-TAATCTACTCTCTGTCCCTA-3'	62.0	1014
	c	14334-14318	*Reverse*	5'-GCTCAGAGCCAGCAGAA-3'		
Int.1/Exon 2/Int.2		20301-20320	*Forward*	5'-AACCGTTCTCTTGCAATCCA-3'	61.5	449
	c	20749-20731	*Reverse*	5'-ACCTGAGCCAGAACTGTTT-3'		
Exon 3/Exon 4		26997-27017	*Forward*	5'-ACAGGAATGTATGAGAGTTGG-3'	61.5	1880
	c	28876-28857	*Reverse*	5'-CAGACTTCCTGCAATACCAG-3'		
Int. 4/Exon 5/Int. 5		29772-29790	*Forward*	5'-CCAAAGGAAAGTTCCCACG-3'	61.4	494
	c	30273-30254	*Reverse*	5'-GATGACCATGCTAAGAGGTG-3'		
Int. 5/Exon 6/Int. 6		31294-31314	*Forward*	5'-ACACCACATCTCAGCTATATT-3'	60.1	481
	c	31774-31756	*Reverse*	5'-ACAAATGAGGGCTCAGAAC-3'		
Int. 6/Exon 7/Int. 7		32734-32753	*Forward*	5'-GCTTCCTGTTGATCACTAGT-3'	60.5	347
	c	33085-33067	*Reverse*	5'-ACAAAAGGTCAGGGACTG-3'		
Int. 7/Exon 8/Int. 8		33761-33780	*Forward*	5'-ACTCCCCAAACAATAAAGCT-3'	59.0	464
	c	34224-34205	*Reverse*	5'-GGCTCAGAAGGTAAAGAATC-3'		
Int. 8/Exon 9/Int. 9		35403-35422	*Forward*	5'-GAATGGCCTGAGTATAGATA-3'	58.8	381
	c	35783-35763	*Reverse*	5'-CCTGAAGGTTCTTATCACAAG-3'		

c = complementary to