

1 **Genetic variability detected at the lactoferrin locus (*LTF*) in the Italian Mediterranean**
2 **river buffalo**

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8 Short title: Genetic variability at the river buffalo *LTF* gene

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1 **Abstract.** Lactoferrin (*LTF*) is multi-functional protein belonging to the whey protein fractions of
2 the milk. The gene *LTF* encoding for such protein is considered a potential candidate for body
3 measurement, milk composition and yield. This study reports on the genetic variability at *LTF*
4 *locus* in the Italian Mediterranean river buffalo and its possible association with milk yield.
5 Eleven polymorphic sites were found in the DNA fragment spanning the exons 15-16. In
6 particular, the intron 15 was extremely polymorphic with 9 SNPs detected, whereas the
7 remaining 2 SNPs were exonic mutations (g.88G>A at the exon 15 and g.1351G>A at the exon
8 16) and both synonymous. The genotyping of the informative samples evidenced 3 haplotypes,
9 whose frequencies were 0.6; 0.3 and 0.1 respectively, whereas the analysis of the exonic SNPs
10 showed a perfect condition of linkage disequilibrium (g.88A/g.1351G and g.88G/g.1351A). The
11 association study carried out by using the SNP g.88G>A showed that buffalo *LTF* gene has no
12 statistically significant influence on daily milk yield. This study adds knowledge to the genetic
13 variability of a species less investigated than the other ruminant species, that may serve as a
14 useful tool for large-scale screening of buffalo populations.

15

16 **Additional keywords:** *Bubalus bubalis*, lactoferrin, polymorphism, haplotype, milk yield,
17 marker assisted selection

18

1 **Introduction**

2 In Italy there is a buffalo industry of limited size, in comparison with many east Asian countries,
3 but of great economic relevance due to the production of Mozzarella PDO (Protected
4 Denomination of Origin - Reg. EC 510/2006) one of the most popular and spread Italian dairy
5 product. River buffaloes farmed in Italy are named 'Mediterranean Italian', aiming at
6 distinguishing them from other European populations which are not at the same genetic level
7 (Borghese, 2005).

8 In 2012, the consistency of the Italian buffalo stock was about 387,000 heads, with an
9 increase of 7.2% over the previous year. The production of buffalo milk in 2012 amounted to
10 1,924,553 tons, with an increase of 7.79% compared to 2010 (<http://www.aia.it>). Also an
11 improvement in buffalo milk composition occurred in the last few years, with the average protein
12 and fat content moving from 4.65% and 8.10% in 2003 to 4.70% and 8.30% in 2012,
13 respectively, (<http://www.anasb.it/home.htm>). These figures could be ascribed to the effort that
14 has been undertaken in improving the buffalo management system, feeding and also the breeding
15 programme.

16 Advances of molecular genetics offer the possibility to investigate genomic regions that
17 affect traits of economic importance and to identify useful genetic polymorphisms for marker-
18 assisted selection (MAS) programmes. In recent years, some studies have been carried out on
19 buffalo for the identification of genetic polymorphisms at milk protein *loci* (Masina *et al.* 2007;
20 Cosenza *et al.* 2009a, b; Pauciullo *et al.* 2011; Cosenza *et al.* 2014), and candidate genes
21 responsible for the variation of the quali-quantitative characteristics of the Mediterranean water
22 buffalo milk were identified (Cosenza *et al.* 2007; Pauciullo *et al.* 2010a), as well as significant
23 associations with milk yield (Pauciullo *et al.* 2012a, b) and milk coagulation properties (Bonfatti
24 *et al.* 2012) have been found.

1 Lactoferrin (*LTF*) is an iron-binding glycoprotein and it belongs to the whey protein
2 fractions of the milk. It is a multi-functional protein playing an important role in antibacterial,
3 antiviral, antifungal, anti-inflammatory, anti-oxidant, immune-modulatory activities and in the
4 regulation of iron metabolism (Vogel *et al.* 2002; Kang *et al.* 2008). Apart from all the
5 aforementioned functions, the efficient expression of lactoferrin as milk component is also very
6 important. In fact, *LTF* is considered a potential candidate gene for body measurement (withers
7 height, body length and chest circumference) as well as for milk composition and yield (Guo *et*
8 *al.* 2010). Lactoferrin concentration is quite variable across different species and in particular in
9 buffalo's milk (0.332 ± 0.165 g/kg) appeared to be lower than that reported in human milk, but
10 higher than found in bovine milk (Giacinti *et al.* 2013).

11 The *LTF* gene has been particularly investigated in cattle and goat and many
12 polymorphisms have been found in these two species (Seyfert *et al.* 1994; Lee *et al.* 1997; Li *et*
13 *al.* 2004; Daly *et al.* 2006; Kaminski *et al.* 2006; Kang *et al.* 2008; Arnould *et al.* 2009;
14 O'Halloran *et al.* 2009; Huang *et al.* 2010; Pauciullo *et al.* 2010b), some of which showed
15 associations with milk production traits and antimicrobial properties (Lee *et al.* 1997;
16 Li *et al.* 2004; Guo *et al.* 2010). In contrast, few studies have been carried out in buffalo.
17 Currently, only short, partial genomic or cDNA sequences, all related to Indian buffaloes, are
18 available in EMBL. Two synonymous SNPs (adenine-guanine transitions) within the exon 16 of
19 *LTF* gene and different polymorphisms in non-coding regions (intron 4 and intron 9) have been
20 found by comparing lactoferrin sequences of different Indian buffalo breeds (Murrah, Jaffarabadi,
21 Toda, Marathwada, Pandharpuri and Mehsana) (Kathiravan *et al.* 2009; Kathiravan *et al.* 2010).
22 Polymorphisms within the promoter region have been also found in different Khuzestan buffalo
23 breeds (Khatibi *et al.* 2013). In contrast, so far no sequence and no polymorphisms are available
24 for the Italian Mediterranean buffalo.

1 Considering the split architecture of the *LTF* locus (17 exons over ~30kb in cattle), the
2 aim of our work was to sequence part of this gene in the Italian Mediterranean river buffalo, to
3 detect genetic variability at the *LTF* gene and to investigate possible associations with milk yield.

4

5 **Materials and methods**

6 *Sample collection*

7 Individual blood samples were collected from 553 Italian river buffaloes belonging to 11 farms,
8 located in Salerno and Caserta province (Southern Italy). Sampling was carried out in
9 collaboration with the Italian National Association of Buffalo Breeders (ANASB).

10

11 *DNA isolation and PCR amplification conditions*

12 DNA was isolated from leukocytes, using the procedure described by Goossens and Kan (1981).
13 DNA concentration and OD_{260/280} ratio of the samples were measured by the Nanodrop ND-
14 2000C Spectrophotometer (Thermo Scientific).

15 The DNA regions of the *LTF* gene spanning from the 15th exon to the 16th exon, including
16 the flanking regions, of ten individual samples randomly chosen were amplified by iCycler
17 (BioRad) using the following primers: (forward) LTF15F: 5'-GCTGATGCAGCCTTCTCT-3';
18 (reverse) LTF16R: 5'-TTTAAACCCACATCACCCCT-3'. The sequences of the two primers
19 correspond to nucleotides 15432-15449 and to the complementary nt 17054-17073, respectively,
20 of the partial sequence of the bovine gene deposited at EMBL (acc. no [Z93399](#)).

21 The 25 µl reaction mix comprised 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-
22 HCl (pH 9.0), 0.1% Triton X-100, 3 mM MgCl₂, 200 nmol of each primer, dNTPs each at 400
23 mM, 2.5 U Taq DNA Polymerase (Promega, Madison, WI), and 0.04% BSA. The amplification
24 program consisted of an initial denaturation at 97°C for 2 min, annealing of primers at 62°C for

1 45 s, and an extension step at 72°C for 2 min; then 30 cycles of denaturation at 94°C for 45 s,
2 annealing at 62°C for 45 s, and extension at 72°C for 2 min (except for the final extension of 10
3 min). PCR products were analysed directly by electrophoresis in 1.5% TBE agarose gel (Bio-
4 Rad, CA, USA) in 0.5X TBE buffer and stained with SYBR[®] green nucleic acid stain (Lonza
5 Rockland, Inc, USA). PCR products were sequenced on both strands at CEINGE - Biotecnologie
6 Avanzate (Naples, Italy).

7

8 *Bioinformatic and statistical analysis*

9 Allelic frequencies and Hardy-Weinberg equilibrium (χ^2 test) were calculated for each SNP.
10 Homology searches, comparison among sequences, and multiple alignments were accomplished
11 using DNAsis-Pro (Hitachi Software Engineering Co., Japan).

12 The intron 15 was analyzed for potential microRNA sequence by using the bovine
13 miRBase database (<http://www.mirbase.org/search.shtml>), whereas the program TFSEARCH
14 (<http://www.rwcp.or.jp/papia/> Heinemeyer *et al.* 1998) was used to identify potential
15 transcription factor binding sites and TargetScanHuman (http://www.targetscan.org/vert_61/) was
16 used to identify microRNA target regions.

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

20 A total of 7,260 records for milk yield measured monthly by the official recording system
21 of the Italian Association of Buffalo Breeders (ANASB) on 1,097 lactations of the 553 buffaloes
22 were used. Data were collected in the period January 2009-September 2012.

23 Associations between *LTF* polymorphism and milk yield was investigated with the
24 following mixed linear model (SAS Institute, Cary NC, USA):

1

2
$$y_{ijklm} = \mu + lc_i + hys_j + DIM_k + LTF_l + DIM_k * LTF_l + a_m(LTF_l) + e_{ijklm} \quad (1)$$

3

4 where

5 y_{ijklm} = record of milk yield;

6 μ = overall mean;

7 lc_i = fixed effect of the i^{th} age-parity, ($i=1, \dots, 56$);

8 hys_j = fixed effect of the j^{th} heard-year-season, ($j=1, \dots, 24$);

9 DIM_k = fixed effect of the k^{th} month of lactation, ($k=1, \dots, 10$);

10 LTF_l =fixed effect of the l^{th} LTF genotype, ($l=AA, AG, GG$);

11 $a_m(LTF_j)$ = random effects of individual cow ($m=1, \dots, 553$) nested within *LTF* genotype;

12 e_{ijklm} =random residual.

13

14 Pairwise comparisons among different levels of fixed effects included in model were

15 performed using a Bonferroni adjusted test.

16

17 **Results and discussion**

18 *Polymorphism detection*

19 In order to detect polymorphisms at the Mediterranean river buffalo *LTF locus*, the DNA region

20 spanning from the last 2 nucleotides of the 14th intron to the first 2 nucleotides of the 16th intron

21 was sequenced for a total of 1505 bp (EMBL acc. no. [HG515533](#)).

22 The DNA fragment sequenced showed a homology of 95, 93 and 91% respectively with

23 cattle (EMBL acc. no. [Z93399](#)), goat (EMBL acc. no. [FJ609300](#)) and sheep (EMBL acc. no.

24 [FJ541507](#)) *LTF* gene. All splice junctions follow the 5'GT/3'AG splice rule.

1 The analysis and the comparison of the obtained sequences showed a total of 11
2 polymorphic sites. Nine of these were localized at the level of the 15th intron (g.299C>T,
3 g.367A>G, g.893A>G, g.1051A>G, g.1110A>G, g.1113A>G, g.1120C>T, g.1127C>T,
4 g.1214C>T), none of which affected canonical splicing sites.

5 Moreover, we detected one SNP in exon 15 (g.88G>A) and another in exon 16
6 (g.1351G>A). Both the exonic mutations were found to be synonymous with no changes in the
7 amino acid sequence: p.Thr582 and p.Lys628 respectively. The enumeration of amino acids starts
8 at the first amino acid of the mature peptide, with number 1 (EMBL acc. no. [AJ005203](#)).

9 The presence of the guanine in position 88 characterizes also other *LTF* sequences of
10 buffalo (EMBL acc. no. [JF825526](#), [AJ005203](#)) and of other ruminants, such as goat (EMBL acc.
11 no [X78902](#), [DQ387456](#), [CHU53857](#)), sheep (EMBL acc. no. [NM_001024862](#), [KC161426](#)) and
12 cattle (EMBL acc. no. [GQ351344](#), [FJ589071](#)), therefore its presence might be indicative of an
13 ancestral condition. Analogously, the presence of guanine in position 1,351 characterizes the
14 sequences relating to other ruminant and non-ruminant species, except for some breeds of India
15 (EMBL acc. no. [JF825526](#), [AJ005203](#), [EU518482](#)) that is characterized by the presence of
16 adenine. However, the same locus was described as polymorphic by Kathiravan *et al.* (2010)
17 which also report a G>A transition. Therefore, this finding seems to confirm the guanine as
18 possible ancestral condition.

19 A comparison of the sequence of the 15th and 16th exons (185 bp and 191 bp, respectively)
20 of the gene that we sequenced with the published sequence of the buffalo *LTF* cDNA (EMBL
21 acc. no. [AJ005203](#)) shows two nucleotide differences located at 15th exon, none of which results
22 in an amino acid substitution: g.58C>T, p.Leu572 and g.140C>T, p.Leu600

23 The sequence analysis for the informative samples showed a condition of linkage
24 disequilibrium for most of the detected polymorphic sites. Only three haplotypes were found: H1

1 (ATGGAAGCCCG), H2 (GCAAGGACCCA) and H3 (GCGGGGATTTA) whose frequencies
2 were 0.6, 0.3 and 0.1 respectively (Fig. 1). (Insert Fig.1 here)

3

4 *Intron 15 analysis for potential microRNA sequence*

5 The analysis of the intron 15 (1,125 bp) showed two mature sequence for the miR-181a (EMBL:
6 HG515533, nucleotides 889-906) and miR-2459 (nucleotides 1076-1094). MicroRNA (miR) are
7 small noncoding RNA that regulate gene expression post-transcriptionally and play a key role in
8 development and specific biological processes. The expression pattern of the miR-181a was
9 already investigated in the bovine mammary gland, however no significant difference were
10 reported at different stages of the lactation cycle (Wang *et al.* 2012). On contrary, miR-2459
11 expression pattern in mammary gland has not been investigated yet. Both microRNA are
12 involved in the innate and adaptive immune response regulating target genes like TRAF6 (TNF
13 receptor associated factor 6), TRAF3 (TNF receptor associated factor 3), TNF, MMD (monocyte
14 to macrophage differentiation-associated), etc... and their presence in *LTF* gene might be
15 connected with the defense mechanism related to this milk protein.

16 The analysis of the regulatory regions upstream and downstream of both miR sequences
17 did not evidence any influence of the detected polymorphisms on putative consensus sequences
18 for transcription factors. However the SNP g.893A>G fell within the mature sequence of the
19 miR-181. Nevertheless, except when conserved sequences in splice sites are changed, the real
20 effects of non-coding polymorphisms cannot be easily predicted.

21

22 *Genotyping of Mediterranean river buffalo LTF alleles*

23 Although the detected polymorphisms in the coding region do not result in amino-acid changes,
24 they can still affect gene function by altering the stability, splicing or localization of the mRNA.

1 In general, synonymous changes are less likely to be associated with functional changes or
2 diseases, and so should be given lower priority for genotyping. Nevertheless, because of their
3 potential effect on mRNA stability, they should have higher priority than polymorphisms that lie
4 deep within introns (Risch 2000). For these reasons we decided to genotype the investigated
5 buffalo population only for exonic SNPs: g.88G>A and g.1351G>A.

6 The genotyping of 553 buffalo DNA samples was performed at the KBiosciences (Herts,
7 UK, <http://www.kbioscience.co.uk>) laboratory. The major allele had a relative frequency of about
8 0.682 in both *loci* and χ^2 values showed that there was no evidence of departure from the Hardy-
9 Weinberg equilibrium ($P \leq 0.05$). A complete linkage disequilibrium was observed for these 2
10 SNPs (average D' and r^2 values were 1.0 and 1.0 respectively), whose haplotype (g.88A/g.1351G
11 and g.88G/g.1351A) frequencies were 0.318 and 0.682, respectively, i.e. equal to allelic ones.
12 According to Gabriel *et al.* (2002), *loci* can be considered as being in the same haplotype block.
13 Thus only one SNP (g.88G>A) was considered in running model (1). Genotype distribution of
14 buffalo cows for the SNP g.88G>A are reported in Table 1. (Insert table 1 here)

15

16 *Association of LTF polymorphism on milk yield*

17 Despite the relatively large amount of data analyzed and the accurate measures on milk yield, the
18 present study suggests that no direct effect of the investigated *LTF* polymorphisms on the
19 production trait studied. The larger difference in the least squares means for milk yield was
20 estimated in 0.36 kg/d between the genotypes AA and AG, whereas the GG showed an average
21 milk production of 8.35 kg/d. These differences were not significant, therefore this SNP appears
22 to be selectively neutral in relation to milk yield. Although not significant, this result adds new
23 information to the recent reports on polymorphisms found in other genes influencing the milk
24 yield in river buffalo (Table 2). In particular, SNPs found in *SCD* (Pauciullo *et al.* 2012a) and

1 *OXT* (Pauciullo *et al.* 2012b) genes were significantly associated with milk production, whereas
2 recently a polymorphism in the α s-1 casein encoding gene (*CSN1S1*) was found selectively
3 neutral in relation to milk yield (Cosenza *et al.* 2014) like observed in the present study for *LTF*
4 (Insert table 2 here). However, this result does not exclude the existence of a association of *LTF*
5 polymorphisms in other buffalo populations. In fact, different breeding conditions might have an
6 indirect effect on milk production, as well as linkage disequilibrium conditions of *LTF* with other
7 genes influencing milk production traits might result in significant associations with milk yield.
8 In this context, the lactoferrin gene in cattle is mapped on chromosome 22q24 at a distance of
9 6.5cM from a QTL for SCS (Heyen *et al.* 1999), whose content was demonstrated to influence
10 the milk yield and its technological properties (Schutz 1994; Ikonen *et al.* 2004; Cassandro *et al.*
11 2008). Furthermore, in the same chromosomal region Ashwell *et al.* (2004) identified a QTL
12 affecting milk production, protein percentage and SCS. In support of this, the buffalo lactoferrin
13 was already positively correlated with total proteins and SCS and negatively correlated with
14 lactose and titratable acidity (Giacinti *et al.* 2013), analogously to what was observed for cattle
15 (Kaminski *et al.* 2006; Parland *et al.* 2010). Therefore further studies are necessary to better
16 verify this link also in a Mediterranean buffalo population as well in other buffalo breeds.

17 In cattle, *LTF* polymorphisms were also associated with other traits, including
18 reproductive performances (O'Halloran *et al.* 2010; Zabolewicz *et al.* 2012). Although it is
19 known that in dairy cows the intense selection for milk
20 production resulted in health and fertility problems, the genetic progress for
21 this trait is the major challenge also in river buffalo. Therefore, the improvement of the
22 reproductive performances could be in the next future also a goal for buffalo selective breeding
23 programmes. In this perspective, the detected polymorphisms at *LTF* locus can represent a useful
24 investigative tool for future association studies. In fact, the protective effects of the correlation

1 with SCS or total milk proteins (Giacinti *et al.* 2013) and the potential role in fertility
2 preservation (O'Halloran *et al.* 2010) could be combined to preserve both productive
3 performances and health of the animals.

4

5 **Conclusions**

6 The polymorphisms detected in the present
7 work represent the first genetic markers for the
8 Mediterranean river buffalo lactoferrin
9 encoding gene since no information were
10 available so far for this breed. The DNA
11 fragment between the exon 15 and 16 resulted
12 extremely polymorphic considering that a total
13 of 11 SNP were found in about 1500 bp.

14 No association between the exonic SNPs
15 g.88 G > A and g.1351 G > A and milk yield was
16 revealed in the present study. Although this
17 result suggests that these polymorphisms are
18 selectively neutral in relation to the
19 improvement of milk production in
20 Mediterranean river buffalo reared in
21 Campania, they add important information in
22 terms of genetic variability and they could be
23 used for future studies of association with other

1 traits of economic interest like protein and fat
2 production, SCS or reproductive traits.

3 Furthermore, considering that the genome
4 wide SNPchip is currently not available for
5 river buffaloes, these SNPs might be
6 considered as potential candidate for the
7 development of this tool and therefore
8 contribute in future genome wide association
9 studies (GWAS).

10

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22

23 **Conflict of interest**

1 The authors declare that they have no conflict
2 of interest.

3

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Table 1. Genotyping data, allele frequency, absolute (relative) frequencies of buffalo cows, lactations and tests across genotypes of the SNPs: g.88G>A SNP at the exon 15 and g.1351G>A at the exon 16 of the *LTF* gene in Mediterranean river buffalo population

Position	Genotype distribution*				Allele freq.*		Absolute and relative frequencies used in the model				
	aa	ab	bb	TOT	a	b	Genotype	n of cows	n of lactations	N of tests	
Exon 15/Exon16	Obs.	253	248	52	553	0,68	0,32	aa	253 (45,75)	501 (45,67)	3,268 (45,01)
								ab	248 (44,84)	488 (44,48)	3,278 (45,15)
	Exp.	257,01	239,97	56,01				bb	52 (9,41)	108 (9,85)	714 (9,84)
	$\chi^2=0,619$							total	553 (100)	1097 (100)	7,260 (100)

* allele a = G and allele b = A for the SNP g.88G>A; allele a = A and allele b = G for the SNP g.1351G>A

Table 2. Comparison among the least squares means of milk yield (kg/d) for the three genotypes at the *LTF* g.88G>A SNP estimated with the model (1) and the milk yield data (kg/d) reported for *SCD* (Pauciullo *et al.* 2012a), *OXT* (Pauciullo *et al.* 2012b), and *CSN1S1* (Cosenza *et al.* 2014).

<i>LTF</i>	MY	SE	<i>SCD</i>	MY	<i>OXT</i>	MY	<i>CSN1S1</i>	MY
GG	8.35	0.08	AA	8.63 ^{ab}	CC	8.73 ^{ab}	CC	7.81
AG	8.19	0.08	CA	8.83 ^a	CT	8.21 ^a	CT	7.92
AA	8.55	0.15	CC	6.60 ^b	TT	10.07 ^b	TT	7.56

^{a,b} Means within columns with different superscripts differ (Bonferroni adjusted $P < 0.05$)

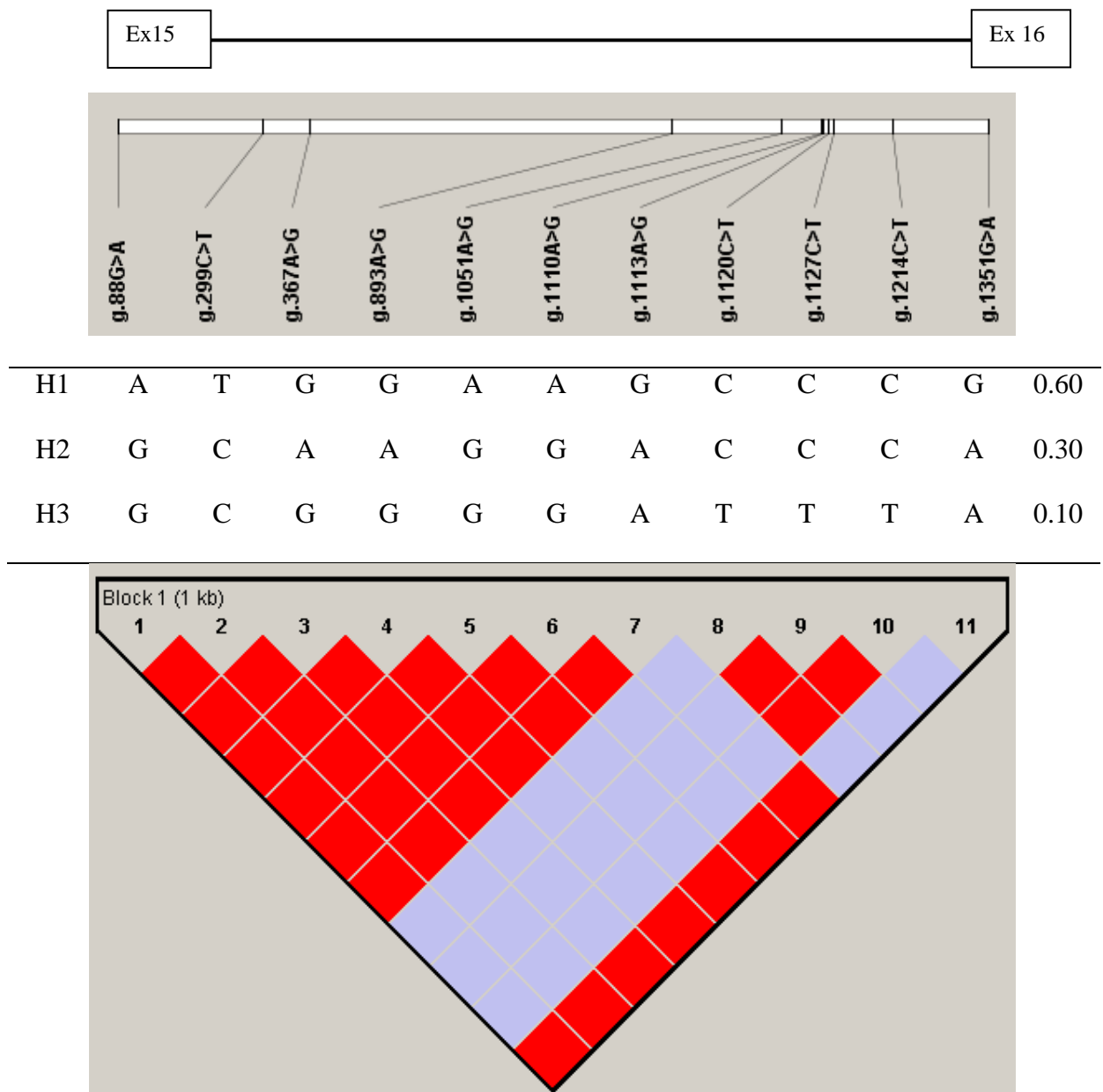


Fig 1. Haplotypes of the *LTF* gene in river buffalo population. Standard color scheme is used to display linkage disequilibrium. Numbers next to each haplotype bar are haplotype frequencies. On top of the figure, a schematic representation of the sequenced regions of the river buffalo *LTF* gene. Exons are indicated by boxes whereas the intron 15 is showed by an unbroken line.