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

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

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

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

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

## Introduction

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

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

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

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

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

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

## Materials and methods

*Sample collection*

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

*DNA isolation and PCR amplification conditions*

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

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

The 25 μl reaction mix comprised 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 9·0), 0·1% Triton X-100, 3 mM $MgCl_2$, 200 nmol of each primer, dNTPs each at 400 mM, 2·5 U Taq DNA Polymerase (Promega, Madison, WI), and 0.04% BSA. The amplification program consisted of an initial denaturation at 97°C for 2 min, annealing of primers at 62°C for

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

*Bioinformatic and statistical analysis*

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

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

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).

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

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

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

where

$y_{ijlmn}$ = record of milk yield;

$\mu$ = overall mean;

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

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

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

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

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

$e_{ijlmn}$=random residual.

Pairwise comparisons among different levels of fixed effects included in model were performed using a Bonferroni adjusted test.

## Results and discussion

*Polymorphism detection*

In order to detect polymorphisms at the Mediterranean river buffalo *LTF locus*, the DNA region spanning from the last 2 nucleotides of the $14^{th}$ intron to the first 2 nucleotides of the $16^{th}$ intron was sequenced for a total of 1505 bp (EMBL acc. no. HG515533).

The DNA fragment sequenced showed a homology of 95, 93 and 91% respectively with cattle (EMBL acc. no. Z93399), goat (EMBL acc. no. FJ609300) and sheep (EMBL acc. no. FJ541507) *LTF* gene. All splice junctions follow the 5'GT/3'AG splice rule.

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

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

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

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

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

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

*Intron 15 analysis for potential microRNA sequence*

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

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

*Genotyping of Mediterranean river buffalo LTF alleles*

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

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

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

*Association of LTF polymorphism on milk yield*

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

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

In cattle, *LTF* polymorphisms were also associated with other traits, including reproductive performances (O'Halloran *et al.* 2010; Zabolewicz *et al.* 2012). Although it is known that in dairy cows the intense selection for milk production resulted in health and fertility problems, the genetic progress for this trait is the major challenge also in river buffalo. Therefore, the improvement of the reproductive performances could be in the next future also a goal for buffalo selective breeding programmes. In this perspective, the detected polymorphisms at *LTF locus* can represent a useful investigative tool for future association studies. In fact, the protective effects of the correlation with SCS or total milk proteins (Giacinti *et al.* 2013) and the potential

role in fertility preservation (O'Halloran *et al.* 2010) could be combined to preserve both productive performances and health of the animals.

## Conclusions

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

No association between the exonic SNPs g.88G>A and g.1351G>A and milk yield was revealed in the present study. Although this result suggests that these polymorphisms are selectively neutral in relation to the improvement of milk production in Mediterranean river buffalo reared in Campania, they add important information in terms of genetic variability and they could be used for future studies of association with other traits of economic interest like protein and fat production, SCS or reproductive traits.

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

## Acknowledgements

This research was financially supported by the Italian Ministry for Agriculture and Forestry Policy-MiPAAF (INNOVAGEN project) and by POR CAMPANIA FSE 2007/2013. Sviluppo di Reti di Eccellenza tra Università – Centri di Ricerca – Imprese. PROGETTO CARINA "Sicurezza, sostenibilità e competitività nelle produzioni agroalimentari della Campania" CUP B25B09000080007

## Conflict of interest

The authors declare that they have no conflict of interest.

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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

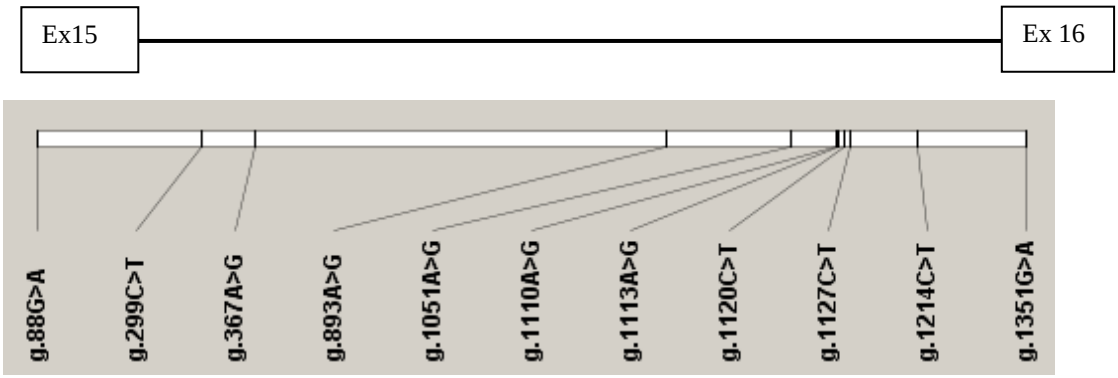
| **Position** | | **Genotype distribution*** | | | | **Allele freq.*** | | **Absolute and relative frequencies used in the model** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | **Genotype** | **n of cows** | **n of lactations** | **N of tests** |
| | | aa | ab | bb | TOT | a | b | aa | 253 (45,75) | 501 (45,67) | 3,268 (45,01) |
| Exon 15/Exon16 | Obs. | 253 | 248 | 52 | 553 | 0,68 | 0,32 | 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).

| ***LTF*** | **MY** | **SE** | ***SCD*** | **MY** | ***OXT*** | **MY** | ***CSN1S1*** | **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 $P<0{\cdot}05$)




| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H1 | A | T | G | G | A | A | G | C | C | C | G | 0.60 |
| H2 | G | C | A | A | G | G | A | C | C | C | A | 0.30 |
| H3 | G | C | G | G | G | G | A | T | T | T | A | 0.10 |

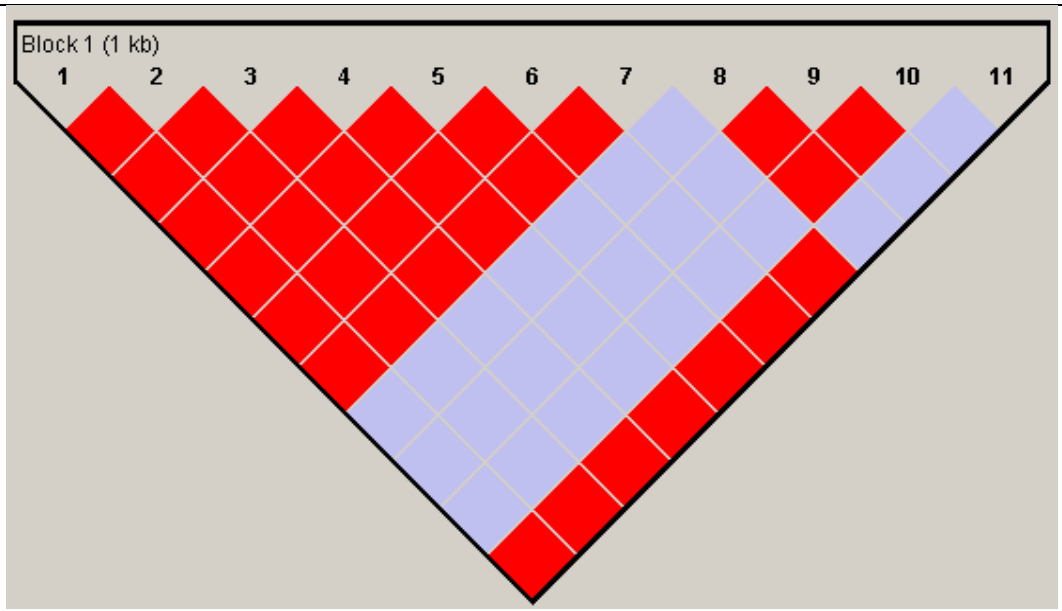



Fig 1. Haplotypes of the *LTF* gene in river buffalo population. Standard color scheme is used to display linkage disequilibrium. Numbers next to each haplotype bar are haplotype frequencies. On top of the figure, a schematic representation of the sequenced regions of the river buffalo LTF gene. Exons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