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A novel polymorphism in the Oxytocin receptor encoding gene (*OXTR*) affects milk fatty acid composition in Italian Mediterranean river buffalo

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Summary

The Oxytocin Receptor, also known as *OXTR*, is a protein which functions as receptor for the hormone and neurotransmitter oxytocin and the complex oxytocin-oxytocin receptor plays an important role in the uterus during calving. A characterization of the river buffalo *OXTR* gene, amino acid sequences and phylogenetic analysis were presented. The DNA regions of the *OXTR* gene spanning exons 1, 2 and 3 of ten Mediterranean river buffalo DNA samples were analyzed and found 7 single nucleotide polymorphisms. We focused on the g.129C>T SNP detected in exon 3 and responsible for the amino acid replacement $CGC^{Arg}>TGC^{Cys}$ in position 353. The relative frequency of T allele was of 0.257. An association study between this detected polymorphism and milk fatty acids composition in Italian Mediterranean river buffalo was carried out. The fatty acid

composition traits, fatty acid classes and fat percentage of 306 individual milk samples were determined. Associations between *OXTR* g.129C>T genotype and milk fatty acids composition were tested using a mixed linear model. The *OXTR* CC genotype was found significantly associated with higher contents of Odd branched-chain fatty acids (OBCFA) (P<0.0006), polyunsaturated FA (PUFA n 3 and n 6) (P<0.0032 and P<0.0006, respectively), Stearic acid (C18) (P<0.02) and lower level of Palmitic acid (C16) (P<0.02). The results of this study suggest that the *OXTR* CC genotype animals might be useful in selection toward the improvement of milk fatty acid composition.

Keywords: Mediterranean river buffalo, *OXTR*, polymorphism, milk fatty acid, association

Introduction

Associations between genetic polymorphisms and dairy traits are of great interest in Buffalo for explaining the mechanisms underlying their genetic variation and for providing useful tools for improving the accuracy and efficiency of traditional selection methods. Among dairy traits, of particular interest is the fatty acid (FA) composition of milk fat. The evaluation of the FA composition in milk from buffalo cows is of extreme importance to human health since high intakes of specific fatty acids such as C 18:2 cis 9- trans - 11 and C18: 1 trans-11 (Vacenic) can help in the diseases prevention and help in maintaining and regulating of metabolism in humans. Variations in levels of unsaturated FA of milk fat of buffaloes are similar to those found in dairy cows of the partum to eighth week of lactation. However, higher concentrations of CLA in milk of buffalo cows bring greater benefits to human health due to higher amounts of fat in milk of these animals (Verdurico et al. 2012). Candidate genes for fatty acid binding, transport, and metabolism in relation to production traits include *FASN* (fatty acid synthase), *DGAT1* (diacylglycerol O-acyltransferase 1), *SCD* (stearoyl CoA desaturase), *ACACA* (acetyl-CoA carboxylase alpha), *BLG*

(beta-lactoglobulin). Significant associations between polymorphisms in some of these genes and milk production or milk fat related traits have been reported in cattle (Zhang et al. 2009, Komisarek et al. 2011, Mao et al. 2012, Matsumoto et al. 2012, Molee et al. 2015), goat (Badaoui et al. 2007, Dixit et al. 2015), sheep (Scatà et al. 2009, Moioli et al. 2013) and buffalo (Vohra et al. 2006, Pauciullo et al. 2012, Cardoso et al. 2015, de Freitas et al. 2016).

A possible candidate for milk fat composition could be also the Oxytocin receptor enconding gene (*OXTR*). It acts as a receptor for oxytocin and the oxytocin-oxytocin receptor complex plays an important role in the uterus during calving. The *OXTR* belongs to the G protein-coupled receptor (GPCR) family and contains seven transmembrane (TM) domains, an extra-cellular N-terminus and an intracellular C-terminus, which are believed to be involved in receptor regulation (Bathgate et al. 1995). The conserved TM domains are involved in the activation and signal transduction of the G protein (Gimpl & Fahrenholz 2001), whereas the masking of G protein binding sites has been associated with changes in the relative orientation of TM domains 3 and 6 (Barberis et al. 1998). The *OXTR* is differentially expressed in various tissues; it is up-regulated in the cows' uterus during estrus and at the end of pregnancy, and it also increases in the mammary gland and during lactation (Gimpl and Fahrenholz 2001). *OXTR* gene maps on BTA22 where nine QTL for FA composition of meat have been found in a Charolais X Holstein cross population (Gutiérrez-Gil et al., 2010). Five out of 9 have been found to influence the content of palmitic (C16:0), palmitoleic (C16:1), oleic (c9 C18:1) and conjugated linoleic (CLA) fatty acids and the sum of total fatty acid (SUMWFA) index. The other 4 QTL identified on this chromosome affected the stearic acid (C18:0) content, the SFA index and the P:S ratio.

To date, the *OXTR* encoding sequences have been reported for mice (GenBank Acc. No. D86599), human (Acc. No. AY389507), swine (Acc. No. NM_214027), sheep (Acc. No. NM_001009752), cattle (Acc. No. AF101724) and water buffalo (Acc. No. FJ917398; XM_006070767). Cattle and buffalo *OXTR* cDNA contains 1176 nucleotides, encoding for 391

amino acids with a molecular weight of 43 kDa and an isoelectric point of 9.253 (Bathgate et al. 1995; Fleming et al. 2006; Arunmozhi et al. 2014).

In human, the gene spans 17 kb and contains 3 introns and 4 exons. The genomic DNA for *OXTR* can be mainly divided into three segments. The segment 1 contains the 5' non-coding region. The segment 2 starts upstream the ATG initiation codon and spans downstream encoding for most part of the receptor protein including the sixth transmembrane domain of the receptor. The segment 3, instead, contains the sequence encoding for the seventh transmembrane domain of the receptor, the C-terminal region and the entire 3' noncoding region, including multiple polyadenylation signals. There are two introns between the three segments. The largest intron (about 12 kb in human and rat), interrupts the coding region between transmembrane domain 6 and 7. Compared to human, the structural organization of the *OXTR* gene seems to be conserved also in the bovine species apart from only one exon upstream of that there is the ATG initiation codon (Gimpl and Fahrenholz 2001; Feng 2000).

Excluding these studies, to our knowledge, no information on genetic variability has been reported so far at the *OXTR locus* and, consequently, no association study with milk production traits has been carried out. This study aims to explore the genetic variability at Italian Mediterranean river buffalo *OXTR locus* and test possible associations between detected polymorphisms and milk fatty acids composition finalized to a genetic improvement of productive efficiency of the species.

Materials and methods

Sample collection

Individual blood and milk samples were collected from 306 Italian river buffaloes belonging to 14 farms, located in Salerno and Caserta province (Southern Italy), with a similar feeding management, based on 60% forage (silage and hay) and 40% concentrates. Sampling was carried out in

collaboration with the Italian National Association of Buffalo Breeders (ANASB). Milk samples were collected over the whole lactation.

Fatty acid composition of milk samples was determined by gas chromatography (GC Turbo 3400 CX, Varian Inc., Palo Alto, CA, USA). Milk fat extraction was performed according to the Röse-Gottlieb method (AOAC, 1990) modified as described by Nudda et al. (2015). The fatty acid methyl esters (FAME) were prepared with a base-catalyzed trans - esterification according to the International Dairy Federation standard procedure (1999). The FAME was separated in a capillary column (CP-select CB for Fame; 100 m×0.32 mm i.d., 0.25-μm film thickness, Varian Inc., Palo Alto, CA). The injector and FID temperatures were 255°C. The programmed temperature was 75°C for 1 min. It was then raised to 165°C at a rate of 8°C/min, maintained at 165°C for 35 min, increased to 210°C at a rate of 5.5°C/min, and finally increased to 240°C at a rate of 15°C/min. The split ratio was 1:100 with He at a pressure of 37 psi as the carrier gas. The fatty acids were identified by comparing retention times of peaks with those of methyl ester standards as reported by Nudda et al. 2011. In addition published isomeric profile were used to identify trans C18:1 and CLA isomers of interest (Griinari et al. 1998; Kramer et al. 2004; respectively).

DNA isolation and PCR amplification conditions

DNA was isolated from leukocyte, 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).

The DNA regions of the *OXTR* gene spanning exons 1, 2 and 3 of ten individual samples, randomly chosen, were amplified by iCycler (BioRad) using primers designed on the complete bovine genome sequence (EMBL acc. no. NC_007320.5) (Table 1).

PCR amplifications were performed according to the following chemical conditions: 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 μM, 2.5 U Taq DNA Polymerase (Promega, Madison,

WI), and 0.04% BSA in a final volume of 25 μl reaction mix. The thermal condition for the amplification of the exon 1 and 3 consisted of an initial denaturation at 97°C for 2 min, annealing at 62°C for 45 s and extension at 72°C for 2 min, followed by 30 cycles at 94°C for 45 s, 62°C for 45 s and 72°C for 2 min (except for the final extension of 10 min). For the amplification of the DNA region spanning the exon 2, the amplification conditions were the same except for the annealing temperature (67°C).

All 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. Homology searches, comparison among nucleotide and amino acid sequences and multiple alignments were accomplished using DNAsis-Pro (Hitachi Software Engineering Co., Japan).

Phylogenetic analysis was performed using the evolutionary distances computed by the maximum composite likelihood method of MEGA 4 software (Tamura et al. 2007). The evolutionary distances were computed using the Poisson correction model and they are in the units of the number of amino acid substitutions per site. A bootstrap analysis based on 1000 iterations was used to evaluate the robustness of the tree. The amino acid sequences for the *OXTR* were obtained from the EMBL sequence database.

Associations between *OXTR* genotype, fatty acid composition traits, fatty acid classes and fat percentage were tested using the following mixed linear model:

$$y_{ijklmn} = \mu + AGE_i + DIM_j + Month_k + SNP_l + HERD_m + e_{ijklmn} \quad [1]$$

where:

y_{ijklmn} = Fatty acid considered in test;

μ = Overall mean;

AGE_i = fixed effect of the age of the *i*th animal at calving expressed in years (6 classes: 1= <4yrs., 2=4 yrs., 3=5 yrs. , 4=6 yrs., 7=5 yrs., 6 = >7 yrs.);

DIM_j = fixed effect of the *j*th stage of lactation (10 levels of 30 days each);

$Month_k$ = fixed effect of the *k*th month of calving (12 levels);

SNP_l = fixed effect of the *l*th SNP genotype;

$HERD_m$ = Random effect of the *m*th herd

e_{ijklmn} = Random residual

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

The average gene substitution effect (α) was calculated as simple phenotypic regression upon the number of C alleles at the *OXTR locus* (0, 1, 2) weighted for the frequency of the phenotypes.

The variance component associated to the *OXTR* genotype (σ^2_{OXTR}) was estimated by running a mixed linear model with the same structure of [1] but with the SNP effect treated as random Then the contribution of the *OXTR* genotype (r^2_{OXTR}) to the total phenotypic variance of the trait considered was calculated as:

$$r^2_{OXTR} = \frac{\sigma^2_{OXTR}}{\sigma^2_{OXTR} + \sigma^2_{HERD} + \sigma^2_e}$$

where (σ^2_{HERD}) and (σ^2_e) are herd and residual variance component estimated with model [1], respectively.

Results and Discussion

Gene characterization and polymorphism detection

The first exon (partial), the entire second exon and the third exon until the stop codon (TGA) of the *OXTR* gene of ten individual samples randomly chosen were amplified and sequenced. The sequences are available in EMBL with the following accession numbers: LT592263, LT592262, LT592264.

The Mediterranean river buffalo *OXTR* gene contains an uninterrupted ORF of 1176 nucleotides, corresponding to a predicted polypeptide length of 391 amino acids similar to those reported in cattle (Bathgate et al. 1995), sheep (Fleming et al. 2006) and Indian water buffalo (Arunmozhi et al. 2014). The predicted molecular weight of the deduced amino acid sequence was 43,169 kDa with an isoelectric point of 9.41. The ORF is characterized by high CG content with an average value of 64.8%.

Exon 1

The DNA sequence spanning the last 631 nucleotides of the exon 1 showed a homology of about 97% with the corresponding bovine sequence (EMBL AF100633) and 99% with the non-annotated genomic bubaline sequence deposited in GeneBank with the access number KI418690.1 (from nt 1902413 to 1903043), which, therefore, we have identified as putative *OXTR locus*. The comparison of obtained sequences showed a total of 5 polymorphic sites: EMBL no. LT592263 g.230G>T, g.522G>T, g.567C>G, g.577G>C, g.579G>T. Since this exon corresponds to the region 5' UT, such mutations do not affect any triplet coding for the protein. The first SNP falls within a portion of exon 1 that appears to be differentially spliced in the bovine (exon 1a, Bathgate et al. 1995; Telgmann et al. 2003, EMBL AJ490937.1). The subsequent comparison with the buffalo genomic sequence (EMBL KI418690.1) allowed to identify a further nucleotide difference:

g.476C>T (Figure 1). The sequence analysis of the informative subjects showed only two haplotypes: a) GGCGG and b) TTCCT.

Exon 2

The DNA region including the second exon of the buffalo *OXTR* gene was sequenced for a total of 1084 bp (EMBL no. LT592262). The nt 157-159 correspond to the triplet ATG coding for the start codon of the translation (Methionine). The entire sequence showed a homology of 99% with the buffalo genome sequence (KI418690.1), as well as with the corresponding bovine sequence (EMBL AF100633).

A similar degree of homology was also observed with the partial sequence of the Indian buffalo *OXTR* cDNA (EMBL FJ917398) and with the partial Italian Mediterranean buffalo cDNA sequence (EMBL XM_006070767.1). In particular, the first comparison showed 6 SNP, three of which are responsible for amino acids changes: $GTG^{Val}>GCG^{Arg}$, $ATT^{Ile}>GTT^{Val}$, $TGG^{Trp}>CGG^{Arg}$ in position 60, 209 and 302, respectively. On contrary, only one silent SNP was detected by the latter comparison with the Italian Mediterranean buffalo cDNA (Figure 1). No polymorphism was detected from the sequences alignment of the 10 subjects examined in this study.

Exon 3

The complete coding region (from the first nucleotide to the termination codon, TGA) of the third exon of *OXTR* gene was sequenced for a total of 248 bp (EMBL no. LT592264). The sequence alignment among the 10 investigated animals showed two SNP. The first (g.129C>T) is responsible for the amino acid replacement $CGC^{Arg}>TGC^{Cys}$ in position 353, whereas the second (g.139C>A^{Gly356}) is a conservative mutation.

The following comparison with the sequences available in database showed that, excluding the species phylogenetical distant like cetaceans and *Ailuropoda melanoleuca* (Giant panda), the presence of a cysteine in position 353 does not characterize any other of the 13 mammalian species

compared. However, it is interesting to point out that in primates, pinnipeds, felidae, canidae, chiroptera and talpidae, the thymine represents always the first nucleotide of the triplet, although in all these species it is replaced by TAC which encodes for the amino acid Tyr.

In regard of the conservative polymorphism, it is remarkable to notice that, with the exception of the Indian buffalo that is characterized by the presence of adenine, the other mammals considered in the present study are characterized by the presence of cytosine.

Additional polymorphisms were detected through the comparison of the sequences available in EMBL (XM_006070767 and NW_005785401.1) related to the cDNA and the genomic DNA of the Italian Mediterranean buffalo, respectively. In particular, the SNP g.222G>A and g.241C>T are responsible for two amino acid changes, Gly^{384}>Arg and Thr^{390}>Met, respectively (Figure 1).

Analysis of the amino acid sequences and phylogenetic analysis

According to Feng et al. (2000), the putative amino acid sequence derived from these exonic sequences confirmed the occurrence of seven hydrophobic transmembrane regions, which are characteristic of a G-protein coupled receptor. In the three extracellular loops (between transmembrane domains II and III, IV and V, VI and VII) little change was found among the species (Figure 2). This finding leads to state that, throughout mammalian evolution, the primary amino acid sequence of *OXTR* was rather conserved to guarantee the further protein structures for the specific ligand-receptor binding properties. In general, little differences were found only at the edges (N- and C-terminus) and at the third intracellular loop (between transmembrane domains V and VI) which likely have less functionality.

The multiple sequences alignment of the amino acid primary structures between the buffalo *OXTR* and the other considered species allowed the construction of the phylogenetic tree. Closely related mammalians were gathered in different clades. In particular, the phylogenetic analysis showed 3 main clades: A, B and C. The first one includes four embranchments: Minke Wale/Orca embranchment, both members of artiodactyl infraorder Cetacea, American Bison/Yak, Italian Water

Buffalo (XP006070829)/Italian Water Buffalo (XM006070767), Indian Water Buffalo/Italian Water Buffalo (present work), cattle and sheep embranchment, all being members of cetartiodactyla. The clade B includes members of Carnivore and the Feral Pig, a member of cetartiodactyla, with a Feral Pig/Giant Panda embranchment, whereas the Clade C includes members of Rodentia, Soricomorpha, Lagomorpha and Chiroptera with a Rabbit/Flying Foxt embranchment (Figure 3).

These data indicate a greater phylogenetic affinity among artiodactyla compared to that found with others orders. In particular, the Italian buffalo investigated herein showed a higher affinity with the Indian buffalo than the same Italian buffalo already present in database.

Fatty acids composition profile

The average fat content (%), the FA composition (%) and and FA classes (%) are reported in the Table 2. Saturated FA (SFA) represented 71.6% of total FA ranging from 57.9% to 85.9% and C16:0 and C18:1 c9 were the most represented (34.8% and 19.1%, respectively). Monounsaturated FA (MUFA) were 25.17% of total milk FA ranging from 12.6% to 37.4% and the C18:1 c9 represents the majority (76% of total MUFA). The concentration of polyunsaturated FA (PUFA) was 3.21% of the total FA ranging from 1.39% to 5.11%, with the C18:2 n6 predominating (49% of total PUFA), while the C18:3 n3 was present in a lower amount 0.32% of total FA. Trans FA (TFA) represented 1.70% of total FA with the C18:1 t11 the most abundant (59% of total TFA). CLA isomers amounted to 0.76% of total milk FA and the isomer c9, t11 CLA represented the majority (55% of total CLA). The FA profile obtained in this study is typical of animals farmed in intensive systems, with a reduced occurrence of unsaturated fatty acids, compared to graze-based systems.

Results of mixed model analysis did not find statistically significant effects of the age of the buffalo at calving on the fatty acid profile.

Month of calving significantly affected total CLA and TFA ($P<0.05$), evidencing a difference between January and February (data not reported for brevity). DIM affected significantly all the group of FA analyzed ($P<0.01$) denoting a marked lactation curve effects.

Genotyping and association of OXTR polymorphism with milk FA composition traits

It is well known that the cysteine can be easily oxidized to form a dimer containing a disulfide bridge between two cysteines. Such dimer is known as cystine and this feature is very important for the analysis of the primary structure, for effects on changes in secondary structure and for stabilization of the tertiary and quaternary structure of the proteins. Therefore, given the peculiarity of the mutation g.129C>T detected at the exon 3 and the resulting amino acid change ($CGC^{Arg}>TGC^{Cys}$ in position 353), we decided to genotype the investigated buffalo population only for this SNP.

The genotyping of 306 buffalo DNA samples was performed at the KBiosciences (Herts, UK, http://www.kbioscience.co.uk) laboratory. The major allele had a relative frequency of 0.743 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.129C>T is reported in Table 3.

Results of model [1] showed statistically significant effects of the SNP g.129C>T at the *OXTR locus* on the milk FA composition traits. In particular, the CC genotype was associated with higher contents of odd branched-chain fatty acids (OBCFA) ($P<0.0006$), polyunsaturated FA (PUFA n 3 and n 6) ($P<0.0032$ and $P<0.0006$, respectively), and Stearic acid (C18) ($P<0.02$). In general, the estimated allele substitution effects (α) for these FAs indicate a positive percentage improvement with the increase of the number of C alleles. On the other hand, TT genotype animals showed higher level of Palmitic acid (C16) ($P<0.02$), confirmed also by a negative α value (-0.732). Table 4 showed the simple statistics of the FA composition traits along with the genotype effects and allele substitution effects, including only significant associations. The contribution of the *OXTR*

locus to the phenotypic variance of the considered traits was about 0.05 for the FA classes but lower for the single FA (Table 4).

It is well known that n-3 FAs have important roles in the modulation and prevention of coronary heart disease. In fact, PUFA n-3 consumption lowers plasma triglycerides, resting heart rate, and blood pressure and might also improve myocardial filling and efficiency, lowers inflammation, and improves vascular function (Mozaffarian & Wu, 2011). Similarly, PUFA n-6 regulates a wide variety of biological functions, which range from blood pressure and blood clotting to the correct development and functioning of the brain and nervous system (Wall *et al* 2010). In addition, they have important roles in immune regulation and inflammation (Calder 2009). Regarding OBCFA, a potential antitumoral activity on human breast cancer cells has been reported (Wongtangtintharn et al., 2004). About Palmitic acids, which is the most abundant fatty acid of milk and dairy products, has been reported that replacing palmitic acid with oleic acid lowered total and LDL cholesterol concentrations, and the LDL:HDL ratio in humans (Kien et al., 2014), even if its detrimental properties has been recently reappraised (Odia et al., 2015).

Stearic acid has not been shown to elevate blood cholesterol and is rapidly converted to oleic acid in vivo (Bonamone & Grundy 1988). In view of these observations, and of the results of this study we can suggest that the *OXTR*-CC genotype animals might be useful in selection toward increasing of OBCFA, polyunsaturated FA (PUFA n-3 and n-6), stearic acid and decreasing palmitic acid content in milk of Mediterranean River buffalo. Nevertheless, further studies are needed to confirm this.

Conclusions

In conclusion, the identified SNP is the first report to help in understanding the genetic structures of oxytocin receptor gene (*OXTR*) on fatty acid compositions in Mediterranean river buffalo. So far, polymorphisms of *OXTR* have been hypothesized to be correlated only with social behavior related phenomena in humans and other mammals. The SNP g.129C>T at the buffalo *OXTR locus* was

found to have a strong association with composition of several fatty acids of milk, in particular with higher contents of OBCFA, polyunsaturated FA (PUFA n 3 and n 6), Stearic acid (C18) and lower level of Palmitic acid. All these variations might help in producing “healthy” milk. Furthermore, with PUFA and stearic acid influencing meat traits, such as taste and texture, this SNP might even be associated with beef quality. The exact molecular and physiological mechanisms underlying the association of SNP with fatty acid content reported in this study are -so far- unknown and further studies need to deeply investigate this aspect. Nevertheless, the use of this polymorphism as genetic markers can be a useful tool for selection of animals and improve milk quality in Mediterranean river buffalo. This study provides useful genetic information regarding the relation between *OXTR* and fatty acid composition.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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Table 1. Oligonucleotide primers sequence and positions

	Position nt		Primers sequence (5'-3')*	EMBL	TM
Exon 1	18277828-18277847	Forward	TCCCTCTTGCTGCTTTAAGT	NC_007320.5	62 °C
	Complementary to: 18278599 - 18278581	Reverse	ACAACCGCTTCTTTGCCTC		
Exon 2	18279074 - 18279090	Forward	GCCCTCGGGGTTTGTTT	NC_007320.5	62 °C
	Complementary to: 18280265 - 18280249	Reverse	CACGAAGCCCGCCAAAC		
Exon 3	18287534-18287556	Forward	TGATGCCGTGTTCTCCTTTCTTC	NC_007320.5	67 °C
	Complementary to: 18287926-18287903	Reverse	GGGTACAAACTGAAAGGTACCTTA		

* Primers were designed by means of DNAsis-Pro (Hitachi Software Engineering Co., Japan)

< Exon 1

GCTTCGTGGGAACCGGCAAGACCGGCCTTCCGCGCCTCCTAGCGGATCTATGTGGTGGTACAGCCTACGCTGCACGCTGCAGAAAAGCTGGCCACCGGCA

GGCATTGCCGCATCCTAACGCCGTCCGCGCGCATAGCCTCGACAGGGCTCCTTGATCGCTTCTAGTCCCTACCCAGCCACCAGTCAGGGCTGCAGGCGAG

GTGATTCCACCCCAGGTTACAGCGTCCGAKCCCTTAGCATCCTATCAGACTGGGTGCCGGCAGCAGCCACTTCAACCTGCCCCGGGAGCACACGCGTCTT

---***G***---

TGGAACCATCCATGGCGGTGCGACTTCCCCAGGGAACCAAGTGTAGTTTCGCCCTACGACTCGGTTCAGGTAGCTGGATCCAGCGCATGAGTGGACAGGA

TGTTTGGCGCTGGTGGAGCCACTGGGACCCGAGAGAAGGTGTTGGAGTGCTTTGAGATCCTAGTTCCGGAGGCGCCGCCGGTACTCTGAGAGATTGTAAA

---T---

GTACCTGCTCAGGATCTCGAAGGAGATCCCTTGGCTGTCGGGGGCCAAGACKCCTGTTGAGTTCCCSAACCTTCGASGKTGCTGGGCTGGGGAGTATCTC

---T---------G---------C-T---

Exon 2

CCCCGAGAGGTGTCAGGGGTACTGAGAACAGAGTCCGACCCAGCAGATCGGTCCTCGGAGTCTCAGGGAGGGGAACCCAGGAGCCCCCACCCCGCCTCCA

M[1] E G A

GGCACGCCGGGACTGCAAGTCGGGCCCCGCCCCCGCGCCGCGCCTTAAAGGACTCGAAGGCCGGGGCGCACCGCGGCGGCCACGGTC**ATG**GAGGGTGCGT

--------C----

F A A N W S A E E V N G S A A P P G T E G N R T A G P P Q R N E A L

TTGCGGCAAACTGGAGCGCTGAGGAGGTCAACGGGAGCGCGGCGCCGCCGGGAACCGAGGGCAATCGCACTGCCGGGCCGCCACAGCGCAACGAGGCCCT

---C---

A R V E V A V L C L I L F L A L S G N A C V L L A L R T T R H K H

GGCGCGGGTGGAGGTGGCCGTGCTGTGCCTCATCCTGTTCCTGGCGCTGAGCGGCAACGCGTGCGTGCTGCTGGCGCTGCGCACCACGCGCCACAAGCAC

---C---

A[60]

S R L F F F M K H L S I A D L V V A V F Q V L P Q L L W D I T F R

TCGCGCCTCTTCTTCTTCATGAAGCACCTGAGCATAGCCGACCTGGTGGTGGCGGTGTTCCAGGTGCTGCCGCAGCTTCTGTGGGACATCACGTTCCGCT

F Y G P D L L C R L V K Y L Q V V G M F A S T Y L L L L M S L D R C

TCTACGGGCCCGACCTGCTGTGCCGCCTCGTCAAGTACCTGCAGGTGGTGGGCATGTTCGCGTCCACCTACCTGCTGCTGCTCATGTCGCTCGACCGCTG

L A I C Q P L R S L S R R T D R L A V L V T W L G C L V A S A P Q

CCTGGCCATCTGCCAGCCGCTGCGCTCGCTGAGCCGCCGCACCGACCGCCTGGCGGTACTCGTCACATGGCTCGGCTGCCTGGTGGCCAGCGCGCCGCAG

---T---

---T---

V H I F S L R E V A D G V F D C W A V F I Q P W G P K A Y I T W I

GTGCACATCTTCTCGCTGCGCGAGGTGGCCGACGGTGTCTTCGACTGCTGGGCCGTTTTCATTCAACCCTGGGGGCCCAAAGCCTACATCACGTGGATCA

---G---

T L V V Y I V P V I V L A T C Y G L I S F K I W Q N L R L K T A A A

CGCTCGTCGTCTACATTGTGCCCGTCATCGTGCTTGCCACCTGCTATGGCCTTATCAGCTTCAAGATCTGGCAGAATTTACGGCTCAAGACGGCGGCGGC

----------G---

V[209]

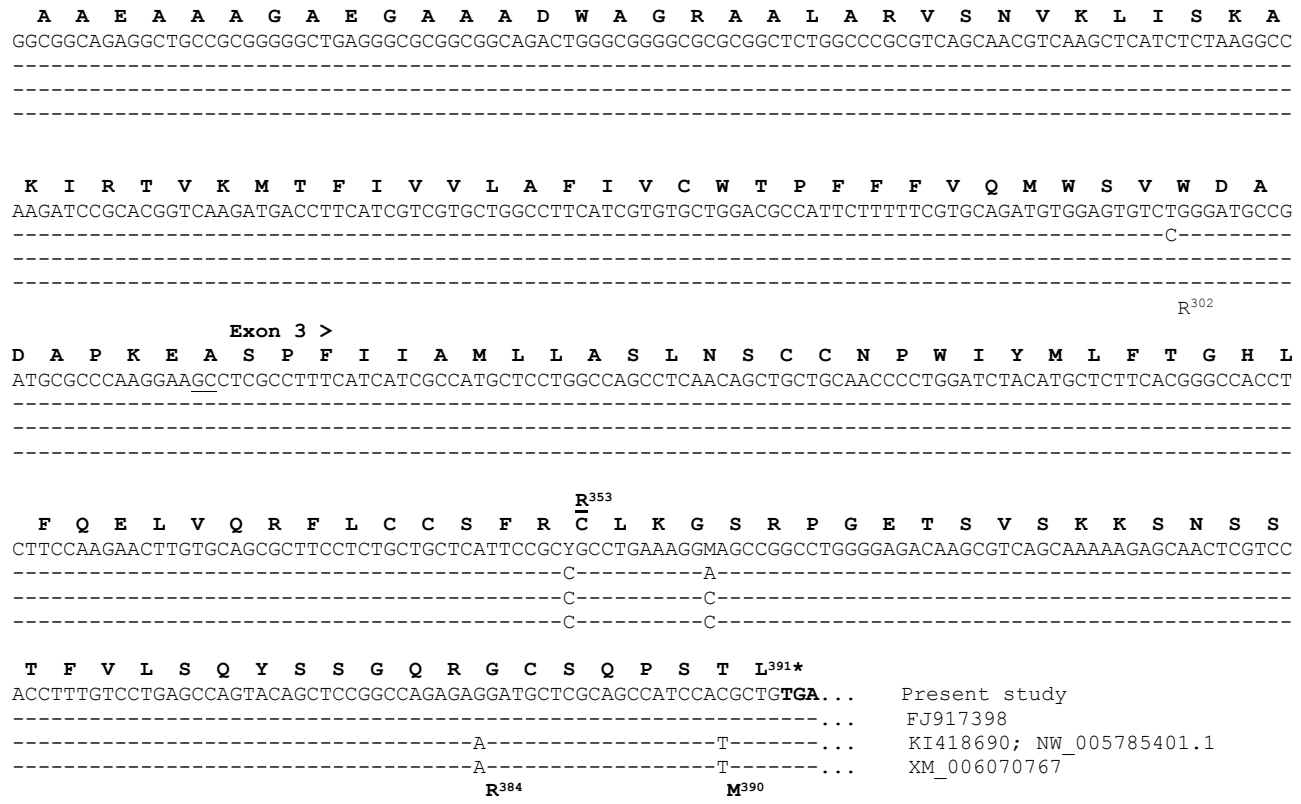

Figure 1. Alignment of the assembled Italian Mediterranean buffalo *OXTR* exonic sequences (present study) with sequences available in EMBL related to the Indian buffalo *OXTR* cDNA (FJ917398), Italian Mediterranean buffalo *OXTR* cDNA (KI418690.1), non-annotated genomic bubaline sequence (NW_005785401.1) and genomic DNA (XM_006070767). Alignment was performed using DNAsis pro Software v2.0 (Hitachi). The sites of exon-exon junctions are underlined. In bold type the portion of exon 1 that appears to be differentially spliced in the bovine (exon 1a). Dashes indicate identical nucleotides. The stop codon is symbolized by *. Amino acid numbering is relative to the first aminoacid (Met) of the deduced encoded protein.

```
                                                                  *************************           ******
Italian Water Buffalo       MEGAFAANWSAEE VNGSAAPPGTEGNRTAGPPQRNEALARVEVAVLCLILFLALSGNACVLLALRTTRHKHSRLFFFMKH predicted, present work
Italian Water Buffalo       ------------ ------------------------------------------------------------------
Indian Water Buffalo        ------------ ----------------------------------------------A-------------------
Italian Water Buffalo       ------------ ------------------------------------------------------------------ predicted
Cattle                      ------------A ------------------------------------------------------------------
Yak                         ------------A ------------------------------------------------------------------ predicted
Sheep                       ------------A ---------------------------------S--------------------------------
American Bison              ------------A ------------------------------------------------------------------ predicted
Orca                        ----L-------A ---------A---L-----------------------------------------R---------- predicted
Minke Whale                 ----L-------A ---------A-------------------------------------------Q---------- predicted
Giant Panda                 ----LV------A ----E---EA------------------------------------------------------- predicted
Domestic Dog                --R-L-------A G---E-A-AAQ------------------------------------------------------
Domestic Cat                ----L-------A ----E----A------------------------------------------------------- predicted
Feral Pig                   ---VL-------A --S-----EA------------------------------------------------------
Human                       ----L-------A A-A------A---------R-----------------L-----------------Q----------
Brown Rat                   ---TP-----V-L DL--GV---E------------------------------------------------------
Black Flying Fox            ----LL----V-A -----P--VV---L-------------------S------------------I-------------- predicted
European Rabbit             ----LV----T-A -GA--VS---TE-G-V----------------------------V---------------------
Cape golden mole            ----L-------AAI-A-V-L------L-----R------------------------------------------------ predicted


                            *****************           **********************                 *******
Italian Water Buffalo       LSIADLVVAVFQVLPQLLWDITFRFYGPDLLCRLVKYLQVVGMFASTYLLLLMSLDRCLAICQPLRSLSRRTDRLAVLVTW
Italian Water Buffalo       ----------------------------------------------------------------------------------
Indian Water Buffalo        ----------------------------------------------------------------------------------
Italian Water Buffalo       ----------------------------------------------------------------------------------
Cattle                      ----------------------------------------------------------------------------------
Yak                         ----------------------------------------------------------------------------------
Sheep                       ------A---------------------------------------------------------R---------A--
American Bison              ----------------------------------------------------------------------------------
Orca                        ------------------------------------------------------------V-R---------A--
Minke Whale                 ------------------------------------------------------------V-R--A------A--
Giant Panda                 ------------------------------------------------------------A-R--A------A--
Domestic Dog                ------------------------------------------------------------A-R--A------A--
Domestic Cat                ------------------------------------------------------------A-R--A------A--
Feral Pig                   ------------------------------------------------------------A-R-PA------A--
Human                       ------------------------------------------------------------R---------A--
Brown Rat                   ------------------------------------------------------------R---------G--
Black Flying Fox            ------------------------------------------------------------A-N--A------A--
European Rabbit             ----------------------------------------------M-------------R---T-R---------A--
Cape golden mole            ------------------------------------------------------------R--A------A—
```

```
                          **************                          **********************
Italian Water Buffalo     LGCLVASAPQVHIFSLREVADGVFDCWAVFIQPWGPKAYITWITLVVYIVPVIVLATCYGLISFKIWQNLRLKTAAAAAEA
Italian Water Buffalo     ----------------------------------------------------------------------------------
Indian Water Buffalo      ----------------------------------------------V-----------------------------------
Italian Water Buffalo     ----------------------------------------------------------------------------------
Cattle                    ---------------------------------------------A------------------------------------
Yak                       ---------------------------------------------A------------------------------------
Sheep                     ---------------------------------------------A----------A-----------------E------
American Bison            ---------------------------------------------A--I---------------------------------
Orca                      ---------------------------------------------A------L---A-----T------------------
Minke Whale               ---------------------------------------------A----------A----------T---------P--
Giant Panda               ---------------------------------------------A----------A----------
Domestic Dog              -------------------------------------------V-----S----------A----------------------
Domestic Cat              ---------------------------------------------A----------V----------------------G
Feral Pig                 ---------------------------------------------A----------A------------------E----
Human                     ---------------------------------------------A-----------------------------------
Brown Rat                 -------------------------------------------V-----A----------A---------------------- -
Black Flying Fox          -------V------------E-----------------V--------I-------A-----C------F--------V-
European Rabbit           --------------------E-----------------V-----A--I-------A-----------------L----A-
Cape golden mole          -------V-------------------------------V-----A--I---F---A----------------------DT


                                                    ******************              *********
Italian Water Buffalo     AAGAEGAAADWAG   RAALARVSNVKLISKAKIRTVKMTFIVVLAFIVCWTPFFFVQMWSVWDADAPKEASPFIIAMLLA
Italian Water Buffalo     -------------   ------------------------------------------------------------------
Indian Water Buffalo      -------------   ----------------------------------------------R-------------------
Italian Water Buffalo     -------------   ------------------------------------------------------------------
Cattle                    ------E------   --I---------------------------------------------------------------
Yak                       ------E------   --I-T-------------------------------------------------------------
Sheep                     ----------C--   ------------------------------------------------------------A--------
American Bison            ------E------   --I---------------------------------------------------------------
Orca                      ---P------C--   -G------S-----------------I----------------------E------A--------
Minke Whale               ---P----V-S--   HG------S-----------------I----------------------E------A--------
Giant Panda                               -------S-----------------I----------------------N------A--------
Domestic Dog              -E-R---     -   --------S-----------------I-----------------------------A--------
Domestic Cat              QE--  ---GS--   --------S-----------------I----------------------N------A--------
Feral Pig                 I--T-----GSR-   --------S-----------------I-----------------------------A--------
Human                     -E-P-----GDG-   -V------S-----------------I----------------------N------A---V----
Brown Rat                 -E-NDA-G-  --   --------S-----------------I----------------------VN------A--------
Black Flying Fox            -P-----SSGE   -------------------------I-------------------------T---A--------
European Rabbit           -GPEGA---AA--GAE--------S-----------------I-----------------------------A--------
Cape golden mole          -T-GV-          LTP-----S-----------------I-----------------------------A--------
```

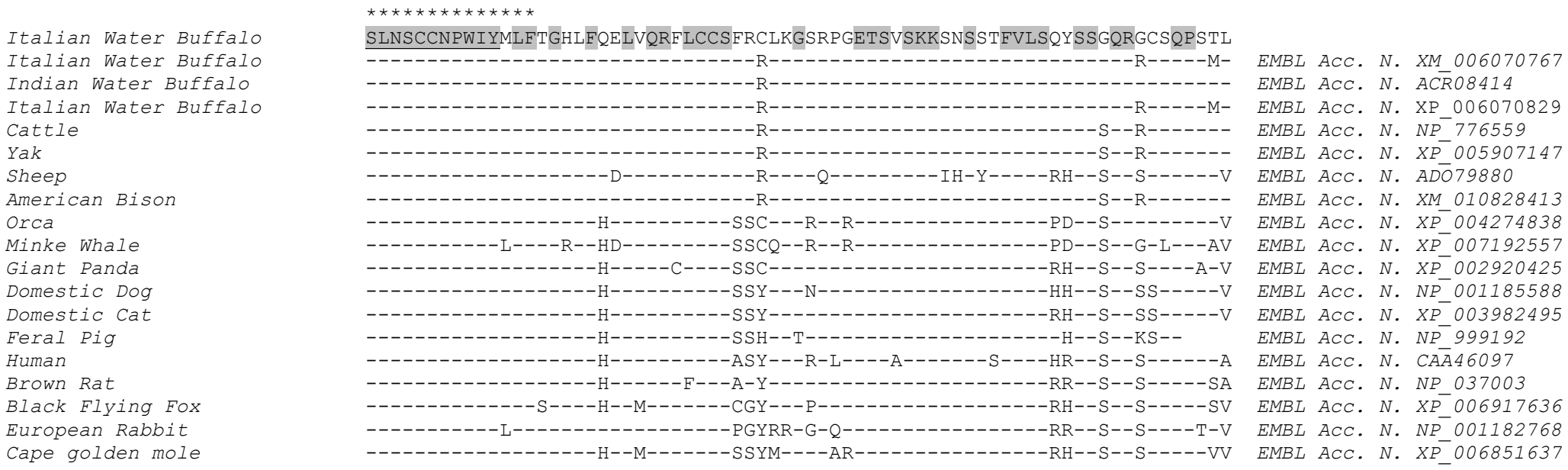

Figure 2. Comparison of protein-coding regions of the water buffalo *OXTR* with cattle, yak, sheep, bison, orca, minke whale, giant panda, dog, cat, pig, human, rat, flying fox, rabbit and cape golden mole. Accession numbers of sequences compared is shown in the figure. Dashes (–) indicate the identical amino acid residues. Amino acids full matching the buffalo sequence of the present work are shown as shaded. The protein portion corresponding to the 7 transmembrane receptor (rhodopsin family) is underlined, whereas the transmembrane (TM) domains are indicated by asterisks (*)

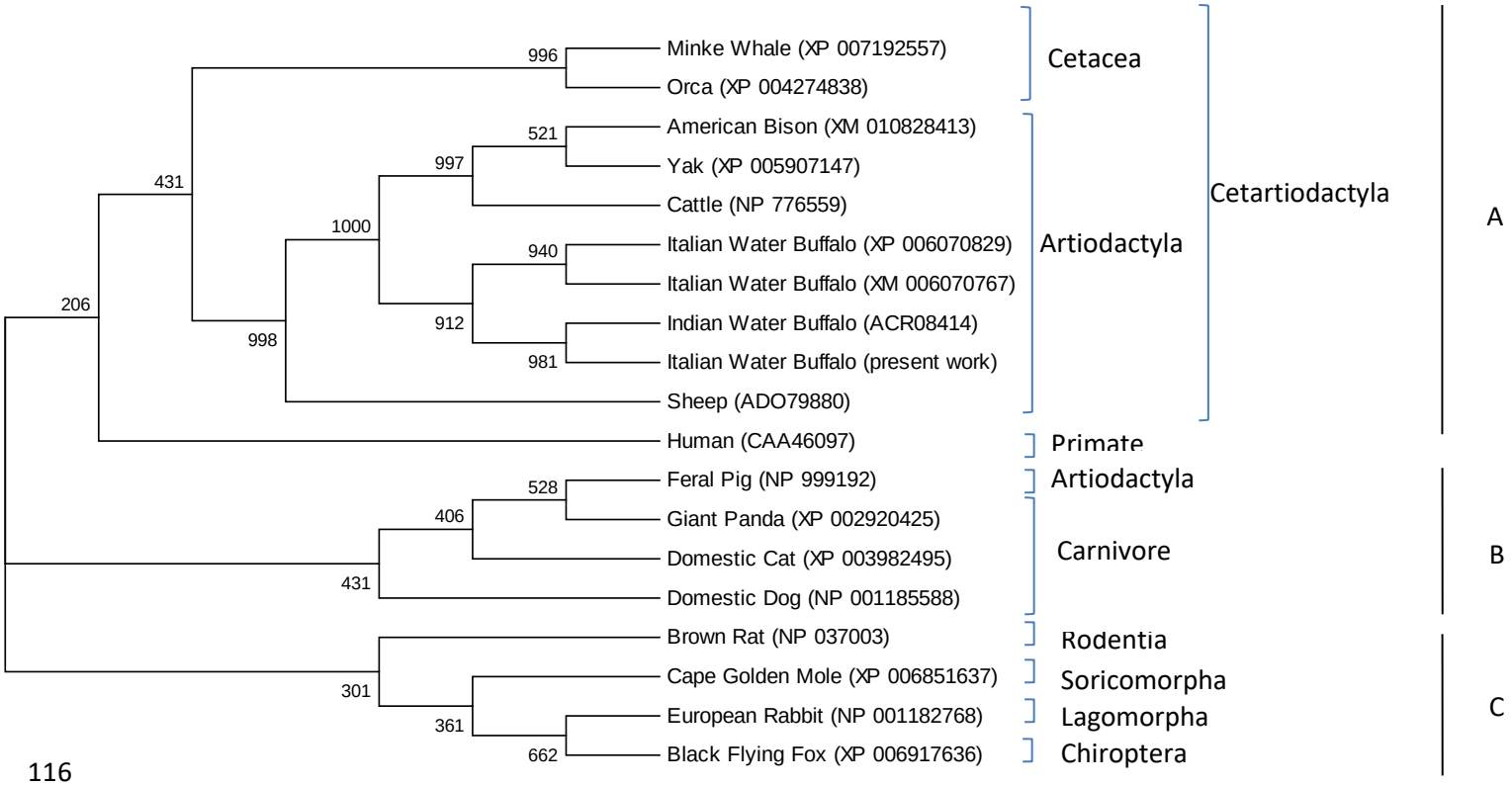

Figure 3. Phylogenetic relationship of the peptide sequence of Italian water buffalo (*Bubalus bubalis*) *OXTR* with other 14 peptide/protein sequences reported in different mammals. The MEGA v4.0 program was used to reconstruct a phylogenetic tree. Bootstrap values are indicated.

122 **Table 2.** Average fat content (%), fatty acid composition (%) and fatty acid classes (%) of 306 dairy
123 buffaloes milk samples measured in 14 herd located in Southern Italy

															P
Herd	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Fat (%)	7.44	7.99	7.94	7.28	7.00	7.80	6.93	8.03	9.34	8.43	8.58	6.24	7.42	7.59	**
Fatty acid (%)															
C4:0	3.47	4.38	3.85	3.90	3.51	4.10	4.35	3.88	3.86	3.65	4.42	3.93	3.80	3.60	**
C6:0	1.61	1.96	2.04	1.86	1.71	1.69	1.83	1.62	1.49	1.52	1.93	1.54	1.64	1.52	**
C8:0	0.8	0.98	1.14	0.95	0.90	0.78	0.86	0.77	0.66	0.73	0.95	0.69	0.79	0.72	**
C10:0	1.73	2.05	2.53	2.03	1.96	1.60	1.77	1.65	1.33	1.47	1.95	1.39	1.65	1.49	**
C12:0	2.38	2.72	3.30	2.63	2.60	2.11	2.34	2.28	1.76	1.96	2.57	1.88	2.22	1.98	**
C14:0	11.07	12.55	12.74	11.65	11.18	10.16	10.81	10.62	9.49	9.26	11.56	9.95	10.50	9.61	**
C14:1	0.73	0.84	0.93	0.59	0.71	0.59	0.85	0.57	0.46	0.50	0.81	0.61	0.56	0.52	**
C15:0	1.26	1.24	1.24	1.23	1.19	1-10	1.28	0.97	0.89	1.00	1.05	1.03	1.09	0.92	**
C16:0	37.68	39.00	33.50	33.93	33.36	33.83	35.65	33.50	34.45	32.17	35.26	33.65	35.21	34.29	**
C16:1	2.31	2.34	2.19	1.57	1.83	1.76	2.52	1.60	1.49	1.55	2.29	1.78	1.60	1.53	**
C17:0	0.55	0.47	0.54	0.57	0.48	0.48	0.56	0.48	0.43	0.50	0.44	0.58	0.48	0.46	**
C17:1	0.23	0.16	0.21	0.18	0.16	0.16	0.24	0.15	0.12	0.13	0.17	0.22	0.15	0.14	**
C18:0	9.07	8.46	9.21	11.54	11.64	11.20	9.72	12.67	14.24	14.22	8.86	12.15	12.14	12.71	**
C18: *t11* (VA)	0.78	0.67	0.77	0.98	0.84	1.11	0.96	0.94	1.15	1.21	0.68	1.05	1.11	1.71	**
CLA c9,*t11* (RA)	0.38	0.33	0.36	0.39	0.38	0.43	0.46	0.36	0.39	0.45	0.35	0.45	0.43	0.67	**
C18:2 *n6* (LA)	1.42	1.28	1.56	1.34	1.95	1.51	1.32	1.36	1.62	2.51	1.58	1.31	1.36	1.55	**
C18:3 *n3* (ALA)	0.33	0.22	0.22	0.19	0.22	0.26	0.19	0.18	0.21	0.41	0.26	0.16	0.22	0.17	**
C20:4 *n6* (ARA)	0.10	0.08	0.13	0.11	0.13	0.09	0.12	0.08	0.07	0.12	0.09	0.12	0.09	0.10	**
C20:5 *n3* (EPA)	0.03	0.02	0.02	0.03	0.02	0.02	0.02	0.01	0.02	0.03	0.02	0.04	0.02	0.03	**
C22:5 *n3* (DPA)	0.04	0.02	0.04	0.04	0.04	0.03	0.03	0.02	0.03	0.04	0.03	0.05	0.04	0.03	**
C22:6 *n6* (DHA)	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ns
Fatty acid classes (%)															
SCFA	7.74	9.50	9.72	8.87	8.22	8.26	8.94	7.99	7.39	7.44	9.37	7.61	7.96	7.40	**
MCFA	58.79	61.80	57.64	54.74	54.11	52.54	57.24	52.74	51.42	49,50	56.47	52.28	54.08	51.54	**
LCFA	33.47	28.70	32.64	36.38	37.67	39.21	33.82	39.27	41.19	43.06	34.17	40.10	37.96	41.06	**
SFA	72.09	76.18	72.96	72.61	71.03	69.18	71.97	70.84	70.75	68.80	71.12	69.22	71.59	69.26	**
MUFA	24.69	21.22	23.86	24.15	25.31	27.43	25.13	26.36	26.13	26.54	25.80	27.61	25.27	26.98	**
PUFA	3.14	2.53	3.07	3.04	3.50	3.16	2.82	2.67	3.02	4.47	2.94	3.02	2.92	3.51	**
OBCFA	4.14	4.01	4.45	3.87	3.73	4.14	4.10	4.01	3.70	4.83	3.78	3.33	3.46	4.03	**
PUFA n3	0.42	0.28	0.29	0.28	0.30	0.33	0.26	0.22	0.26	0.49	0.32	0.26	0.28	0.24	**
PUFA n6	1.64	1.44	1.84	1.56	2.23	1.72	1.58	1.55	1.80	2.80	1.78	1.58	1.58	1.79	**
Ratio and index															
n6/n3	4.25	5.31	6.41	5.64	7.47	5.30	6.26	6.98	7.47	5.70	5.55	6.17	5.61	7.46	**
AI	3.15	3.98	3.33	3.09	2.88	2.61	3.07	2.73	2.62	2.30	2.95	2.51	2.86	2.48	**
TI	2.67	2.97	2.33	2.37	2.04	2.15	2.39	2.05	2.00	1.90	2.32	2.05	2.32	1.96	**

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Table 3. Genotyping data, allele frequency, relative frequencies of buffalo cows of the SNP g.129C>T at the exon 3 of the *OXTR* gene in Mediterranean river buffalo population**.**

Position	**Genotype distribution**					**Allele freq**		**Relative frequencies used in the model**		
		CC	CT	TT	TOT	C	T	**Genotype**	**n. of cows**	**n. of farms**
exon 3	Obs.	173	109	24	306	0,743	0,257	CC	56,53	14
nt 129	Exp.	169,14	116,72	20,14				CT	35,62	
	χ^2=1.339							TT	7,84	
								Total	100	14

Table 4. Least squares Means of the SNP g.129C>T genotypes at the exon 3 of the *OXTR* gene for some FA, estimation of average substitution effects (α) of a thymine for a cytosine, and contribution of the polymorphism to the phenotypic variance (r^2).

		P	CC (173)	CT (109)	TT (24)	α	r^2_{OXTR}
OXTR g.129C>T	OBCFA	0.0006	3.94 ^{A}	3.89 ^{A}	3.57 ^{B}	0.127	0.05
	PUFA n 3	0.0032	0.313 ^{A}	0.285 ^{B}	0.287 ^{AB}	0.019	0.04
	PUFA n 6	0.0006	1.77 ^{A}	1.63 ^{B}	1.70 ^{AB}	0.079	0.05
	C18	0.02	11.13 ^{a}	10.48 ^{b}	10.61 ^{ab}	0.426	0.004
	C16	0.02	34.46 ^{a}	35.25 ^{b}	35.84 ^{ab}	-0.732	0.02