Journal of Dairy Research

cambridge.org/dar

The functions and mechanisms of sequence differences of DGAT1 gene on milk fat synthesis between dairy cow and buffalo

Dinesh Bhattarai[1], Rahim Dad[1], Tesfay Worku[1], Sutong Xu[1], Farman Ullah[1], Min Zhang[1], Xianwei Liang[2], Tingxian Den[2], Mingxia Fan[3] and Shujun Zhang[1]

[1]Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction, Education Ministry of China, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, People's Republic of China; [2]Key Laboratory of Buffalo Genetics, Breeding and Reproduction Technology, Ministry of Agriculture, Buffalo Research Institute, Chinese Academy of Agricultural Sciences, Nanning 53000, People's Republic of China and [3]Renmin Hospital of Wuhan University, Wuhan 430060, People's Republic of China

Research Article

Cite this article: Bhattarai D *et al* (2020). The functions and mechanisms of sequence differences of DGAT1 gene on milk fat synthesis between dairy cow and buffalo. *Journal of Dairy Research* **87**, 170–174. https://doi.org/10.1017/S0022029920000126

Received: 19 August 2019
Revised: 4 September 2019
Accepted: 9 September 2019

Keywords:
DGAT1; K232A; milk fat; plasmid vectors; promoter analysis

Author for correspondence:
Dinesh Bhattarai,
Email: dbhattarai@webmail.hzau.edu.cn

Abstract

In this research communication we describe the DGAT1 sequence and promoter region in dairy cows and buffalo and compare the activities of DGAT1 between the two species in order to increase knowledge of the cause of milk fat variation. pGL-3 basic vectors were used to construct the reporter gene. Based on the predicted promoter region, 4 truncated plasmid vectors were constructed in cow-DGAT1 and 3 plasmid vectors in buffalo-DGAT1. Each reporter plasmid was transfected into the bovine mammary epithelial cell (BMEC), 293T cell, and CHO cells to analyze the activity using Dual-Luciferase Reporter Assay System. The results show that the region between −93 to −556 bp was essential for cow promoter activity while −84 to −590 bp was essential for buffalo promoter activity revealing these regions contain core promoter. The buffalo has higher promoter activity than cow yet it was not statistically significant. Comparison of candidate mutation K232A between cow and buffalo population revealed the presence of both the allelic population in dairy cows (lysine and alanine) however, only K (lysine) allelic amino acid was found in buffalo population. The absence of the alanine allelic population from buffalo explains the higher fat content of buffalo milk.

The promoter area of the gene has particular importance as it may include the region for various transcription binding sites that control the post-translation modification of the gene. RNA polymerase II is the major enzyme to control the transcription mechanism of the gene, which is further related to the identification and classification of the core promoter region in the sequence (Sandelin *et al.*, 2007). Cows and buffalo kept for milk production are a core farming activity in many parts of the world, and the choice between the two is often influenced by milk constituents, especially milk fat, as well as the variable incidence of pathogenic mastitis (Bhattarai *et al.*, 2018). The DGAT1 gene (K232A mutation) is considered to be one of the most important candidate genes for encoding acyl-CoA:diacylglycerol acyltransferase which eventually affects the milk fat content (Cases *et al.*, 1998; Winter *et al.*, 2002; Bovenhuis *et al.*, 2016). The majority of previous research has been done in cattle, and there are few studies of DGAT1 gene in buffalo (Yuan *et al.*, 2007; Cardoso *et al.*, 2015; Freitas *et al.*, 2016*a*; Parikh *et al.*, 2016; Silva *et al.*, 2016). None of these reports mentioned the identification of K232A polymorphism in the buffalo populations that they studied. Since the milk fat content of cow and buffalo differ considerably, DGAT1 gene expression and function in cow and buffalo could be a subject of study. Although few SNPs were identified in the buffalo DGAT1 sequence, as yet no association study has been performed to correlate DGAT1 influence in buffalo milk fat content (Yuan *et al.*, 2007; Cardoso *et al.*, 2015; Freitas *et al.*, 2016*a*; Silva *et al.*, 2016). Differences in the genomic sequence, especially from coding sequence or transcription factors binding sites in the DGAT1 gene, could help to explain the differences in milk fat quantity among these two species.

Postulating that the DGAT1 gene is a candidate gene for milk fat synthesis, the present study is undertaken to determine the gene structure and prevailing differences in DGAT1 in a large set of tropically adapted animals representing diversified Holstein cow and Murrah buffalo breeds. We specifically investigated the sequences of the 5-flanking region and coding region to find out functional variations.

Materials and methods

Animal sampling, DNA extraction, primer design and SNP study

For the initial mutation screening for exon 8 (K232A), a total of 84 blood samples including 41 cows and 43 buffaloes were used and DNA was extracted as described by Bhattarai *et al.* (2017).

Hannah Dairy Research Foundation

CAMBRIDGE UNIVERSITY PRESS

Information from public database of NCBI (http://www.ncbi.nlm.nih.gov), DGAT1 gene referring to Bos_taurus_UMD_3.1.1 (GCF_000003055.6) for cow and UMD_CASPUR_WB_2.0 (GCF_000471725.1) for buffalo was annotated. Primers were designed to amplify the target sequence of DGAT-1 gene. Primer premier 5.0 software was used to designed all the primers (Premier Biosoft International, Palo Alto, CA, USA), which was synthesized by Sangon Biological Engineering Technology (Shanghai, China). PCR protocol was followed as described previously (Bhattarai *et al*., 2017).

Bioinformatics analysis and software used

We used http://www.fruitfly.org/seq_tools/promoter.html for finding possible promoter sequence and http://www.cbs.dtu.dk/services/Promoter/ for predicting transcription start sites in the 5′ UTR and 5′ upstream sequence of cow-DGAT1 and Buffalo-DGAT1 gene (Li *et al*., 2015). CpG Island was predicted with http://www.urogene.org/methprimer/ and http://www.ebi.ac.uk/Tools/emboss/cpgplot/ software package. Transcription factor (TF) binding sites at predicted sequence was analyzed with a variety of tools (http://www.cbrc.jp/research/db/TFSEARCH.html; http://Zlab.bu.e-du/cluster-buster/cbust.html; http://zlab.bu.edu/cluster-buster/cbust.html).

Promoter cloning and generation of luciferase reporter constructs

In order to clone the bovine *DGAT-1* promoter region, we designed gene-specific primers (online Supplementary Table S1) to amplify a 2.0-kb genomic region upstream of the bovine *DGAT-1* gene TSS. For the generation of the luciferase reporter construct, the 2.0-kb bovine *DGAT-1* promoter fragment was excised simply by digestion with KpnI and BgIII (TaKaRa, Dalian, China) and ligated into the pGL3-basic vector digested with the same restriction enzymes (Fig. 2A).

Cell lines, culture conditions, transient transfection and luciferase reporter assay

We used BMEC (bovine mammary epithelium cell line), HEK293T (human embryonic kidney 293T) and CHO (Chinese hamster ovary cell) cells for the culture experiments. Cells were plated at a density of 1.2×105 cells/well in 48-well dishes and 24 h later, they were transfected with the plasmids. Transfection was done in each well of cell plate by adding 450 ng constructs using Lipofectamine 2000 (Invitrogen). Again about, 450 ng recombinant plasmids were co-transfected with 50 ng pRL-TK. We used pGL3-basic as a negative control. Transfection period was 48 h. Finally, the ratio of firefly luciferase light units to Renilla luciferase light units was analyzed which included three independent experiments.

Statistical analysis

One-way ANOVA was used and the unpaired Student's *t*-test was used to detect significant differences ($P < 0.05$). Values were represented as the mean ± SD, and statistical significance was indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Data were representative of at least three independent experiments.

Results and discussion

Structure characteristics and difference screening in cow and buffalo sequence of the bovine DGAT1 gene

The gene was characterized individually in cow and buffalo sequence investigating the mRNA sequence, protein sequence and the components of 5′ upstream and 3′ downstream region. The genetic open reading frame (ORF) trans-skeleton was determined in cow and buffalo-DGAT1 sequence (online Supplementary Fig. S1). The mRNA and interacting protein were compared between cow DGAT1 and buffalo DGAT1 gene (online Supplementary Table S2 and Fig. S2). The mRNA at 102 (C>T) changes the amino acid from Alanine (A) to Valine (V); 123 (T>C) changes Valine (V) to Alanine (A); 707 (G>A) and 708 (C>A) changes Lysine (K) to Alanine (A) and finally 1464 (C>T) changes Alanine (A) to Valine (V). These changes could be responsible for the difference in the protein structure or functions of DGAT1 between cow and buffalo which could be helpful for further analysis.

Characterization of the bovine DGAT1 gene 5′-regulatory region

We used −2000 bp upstream sequences (considering ATG as +1) to predict the possible promoter sequence, transcription start sites, transcription binding sites and CpG Island in cow and buffalo-DGAT1 respectively (online Supplementary Figs. S3 and S4). Our results revealed that the sequences between +16 to +65; −225 to −396 and −991 to −1040 are predicted as possible promoter sequence in cow-DGAT1 gene. Similarly, two regions are predicted as possible promoter sequence in buffalo-DGAT1 sequence viz. (−383 to −432), (−1025 to −1074). There were few regions predicted for CpG Island when we considered −2000 upstream bp sequences.

Single nucleotide polymorphism and comparison

Previous results from various authors revealed the SNP at exon 8 of the cow (K232A) is highly significant to milk fat. In order to know if this exists in cow and buffalo population, we tested the mutation targeting exon 8 and found the mutation was present only in Chinese Holstein bovine population but not in Chinese cross Murrah buffalo breed (Fig. 1D). We used 84 animals (43 cows and 41 buffalo) to find the frequency of the distribution of genotype. We did not find the change in nucleotide at the same identical position in buffalo-DGAT1 gene indicating that not all of the buffaloes (Chinese cross Murrah) have lysine at 232 positions, although about 25% of the cattle have lysine at 232 positions (online Supplementary Table S3).

Construction of plasmid vectors, isolation of promoter region and its comparison in cow and buffalo

Based on the predicted positions of promoter regions and availability of suitable primers, 4 reporter vectors were constructed to test the core promoter activity in cow DGAT1 gene (viz. CV1, CV2, CV3, CV4) and 3 reporter vectors were constructed to test the core promoter activity in buffalo DGAT1 gene (viz. BV, BV2, BV3), (Fig. 2A). The transfection of the corresponding luciferase reporter plasmids into BMEC, 293T and CHO cells, and the results of these analyses show −93 to −556 bp was essential for cow-DGAT1and −84 to −590 bp was essential for the

https://doi.org/10.1017/S0022029920000126 Published online by Cambridge University Press

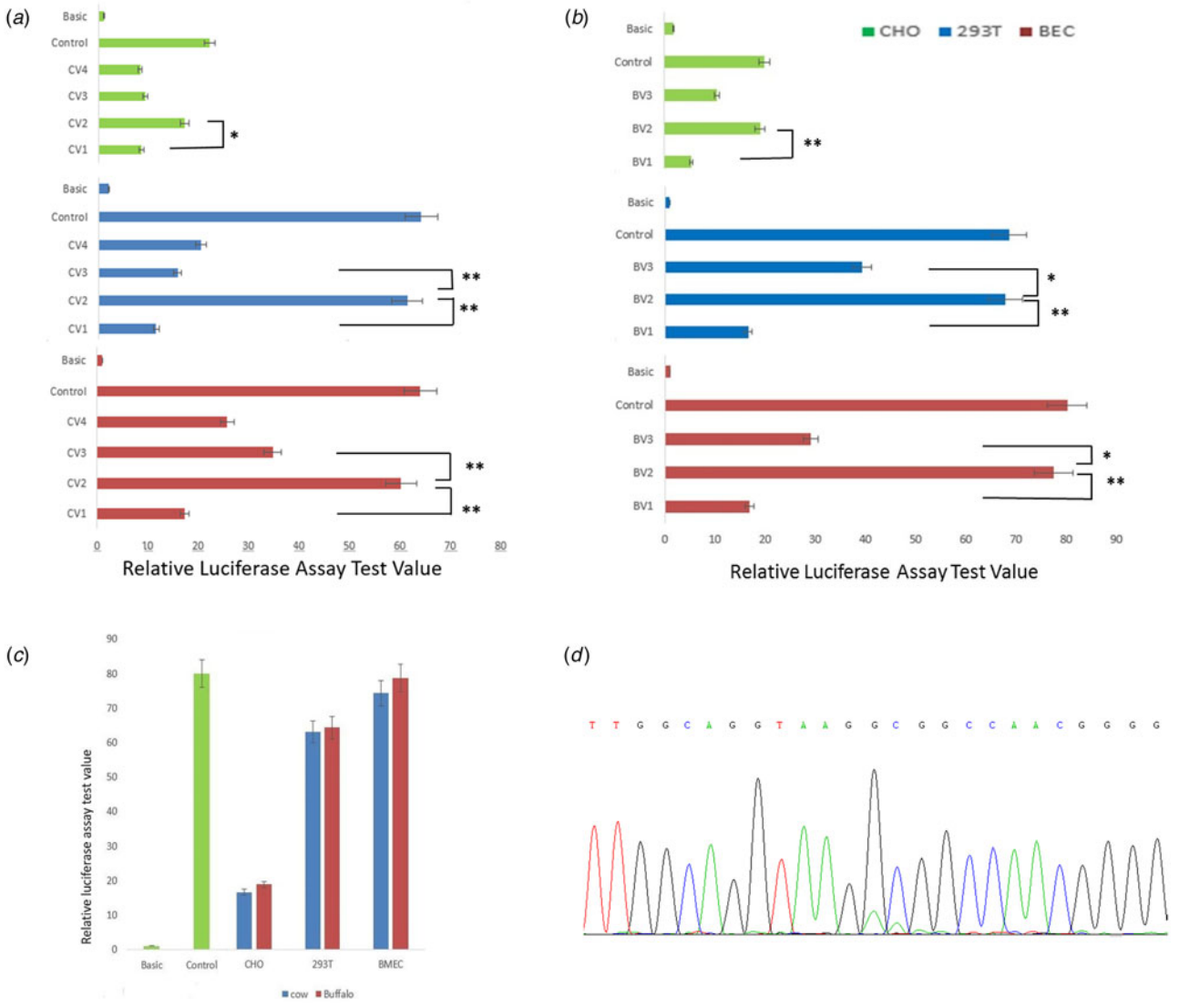

Fig. 1. (A) Identification of the DGAT1 core promoter in cow. (B) Identification of the DGAT1 core promoter in buffalo. (C) Comparison of the identified functional promoter part of cow and buffalo DGAT1 Promoter. Bar represents the relative luciferase activities of the promoter fragments. Values are represented as Mean ± standard deviations. The error bars denote the standard deviation. The unpaired Student's *t*-test was used to detect significant differences. (D) SNP identification at 707 (G>A) and 708 (C>A) position respectively in cow DGAT1 sequence.

buffalo-DGAT1 to maintain the promoter activity (Fig. 1A, 1B). The sequence between −93 to −556 bp region in cow and −84 to −590 bp region in buffalo are highly similar and possess similar transcription binding factors including ATF, AF-2α, NF-1, C-jun, Sp-1 and C-Myb (Fig. 2B). The consensus sequences of ATF, AF-2α, NF-1, C-jun, Sp-1 and C-Myb were predicted by the JASPAR program (jaspar.genereg.net). Though the buffalo DGAT1 promoter activity was higher compared to the cow DGAT1 core promoter, the results were not significantly different. Nevertheless, this difference may go some way to explaining species differences in milk fat, such as the differences in milk fat globule size and composition shown by Ménard *et al.* (2010).

In the mammary gland, the regular lipogenic processes produce TAG and the final stage in this synthetic pathway is catalyzed by DGAT1 (Cases *et al.*, 1998). The genetic polymorphism K232A in the DGAT1 gene exhibits higher milk fat percentage for K allele, and our results revealed that all of the 43 buffalo were KK allelic, whilst 11 cows were KK allelic and 32 cows were AA allelic (online Supplementary Table S3). The previous report analyzed the effect of fat content on DGAT1 (K232A) genotype and found the DGAT1 (KK) allelic population has mean 4.95% of fat content while DGAT1 (AA) allelic population has mean 3.99% fat content in Dutch Holstein-Friesian cows (Argov-Argaman *et al.*, 2013). Comparison of the milk fat content between Jersey and Holstein revealed higher milk fat in Jersey cow (White *et al.*, 2001) and another report of allele distribution between Jersey and Holstein shows, KK (lysine) allele in most of Jersey and AA (alanine) allele in most of Holstein cows (Winter *et al.*, 2002). When we interpret the results of these three studies we can conclude that breeds having KK (lysine) allele have higher fat content in the milk. Allelic determination of buffalo population was done in this study and all the buffaloes were KK allelic in genotype. We can propose that the absence of the alanine allelic population from buffalo explains the higher fat content of buffalo milk and, since segregation of alleles could be important for phenotypic characters in cow and buffalo (Freitas *et al.*, 2016*b*),

https://doi.org/10.1017/S0022029920000126 Published online by Cambridge University Press

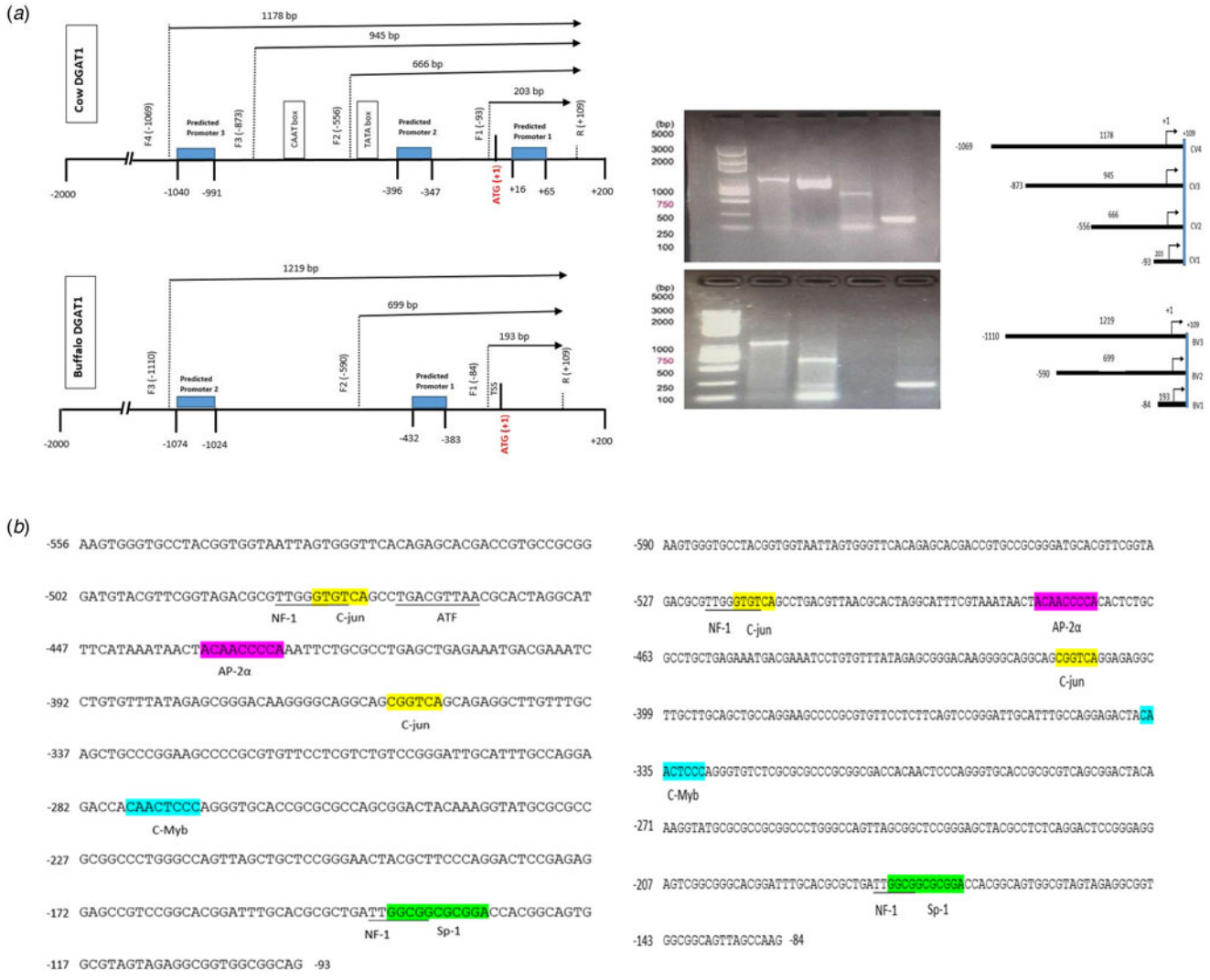

Fig. 2. (A) Predication of the promoter sequence and designed of the primers and PCR amplified fragments of truncated DGAT1 promoter (cow and buffalo) after digestion by KnpI and BglII endonuclease. (B) Sequence and identified TFB sites of the proximal minimal promoter of *DGAT1* gene in cow (left) and buffalo (right). The Putative TFB sites are underlined and colored. http://alggen.lsi.upc.edu/recerca/menu_recerca.html and http://gene-regulation.com/cgi-bin/pub/programs/alibaba2/webbaba2.cgi software was used to predict the possible transcript factors binding sites.

it should be better incorporated to describe the difference in milk fat content.

To understand this phenomenon, the promoter analysis was done in our study. The prediction of the promoter regions and consequent construction of reporter gene to check the activity of promoter area was performed which revealed that the CV2 (−93 to −556) in cow DGAT1 was highly active and the region BV2 (−84 to −590) in buffalo-DGAT1 was highly active compared to other regions that could contain the core promoter. However, the available of similar transcription binding sites in both the expected core promoter regions (Fig. 2B) and the comparison of the strength of promoter activity of CV2 and BV2 failed to demonstrate a significant difference in these two promoters (Fig. 1C). The potential binding motif for the transcription factor Sp-1 is found in repeats in the DGAT1 gene which are termed as the variable number of tandem repeat (VNTR) and the DGAT1 VNTR also showed a strong association with milk fat percentage (Gautier *et al.*, 2007). The allele with 2 repeats affected fat percentage favorably (Cardoso *et al.*, 2015). Similar to the previous report we found the presence of the Sp-1 binding site (Fig. 2B) in the promoter region of both cow and buffalo sequence yet we did not find the repeats as mentioned in previous reports. This could be the reason for the non-significant difference between two identified promoters (for cow and buffalo). The causal agent to cause the K232A variation and activity of K allele for higher fat percent remains hidden and calls for future work.

In conclusion, we have demonstrated the K232A mutation as a candidate marker associated with milk fat. Since all the Chinese cross Murrah buffalo had the K allele and the majority (about 75%) of the Chinese Holstein cow had the A alleles it can be concluded that the presence of the lysine allelic population in buffalo explains the higher fat content of buffalo milk. However, the presence of identical promoter regions in cow and buffalo together with the observation of similar transcription factor binding sites suggests a new avenue for further research to elucidate the core reason behind the difference of DGAT1 expression, function and mechanism in cow and buffalo.

https://doi.org/10.1017/S0022029920000126 Published online by Cambridge University Press

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029920000126.

Acknowledgements. The authors are grateful for financial assistance from the research platform of the Ministry of Agriculture and International Exchange and Cooperation in Science and Technology and EUFP7 projects (PIIFR-GA-2012-912205 and FP7-KBBE-2013-7-613689).

References

Argov-Argaman N, Mida K, Cohen BC, Visker M and Hettinga K (2013) Milk fat content and DGAT1 genotype determine lipid composition of the milk fat globule membrane. *PLoS ONE* **8**, e68707.

Bhattarai D, Chen X, Rehman Z, Hao X, Ullah F, Dad R, Talpur H, Kadariya I, Cui L, Fan M and Zhang S (2017) Association of MAP4K4 gene single nucleotide polymorphism with mastitis and milk traits in Chinese Holstein cattle. *Journal of Dairy Research* **84**, 76–79.

Bhattarai D, Worku T, Dad R, Rehman ZU, Gong X and Zhang S (2018) Mechanism of pattern recognition receptors (PRRs) and host pathogen interplay in bovine mastitis. *Microbial Pathogenesis* **120**, 64–70.

Bovenhuis H, Visker M, Poulsen NA, Sehested J, van Valenberg H, van Arendonk J, Larsen LB and Buitenhuis AJ (2016) Effects of the diacylglycerol o-acyltransferase 1 (DGAT1) K232A polymorphism on fatty acid, protein, and mineral composition of dairy cattle milk. *Journal of Dairy Science* **99**, 3113–3123.

Cardoso DF, de Souza GF, Aspilcueta-Borquis RR, Neto FA, de Camargo GM, Hurtado-Lugo NA, Scalez DC, de Freitas AC, Albuquerque LG and Tonhati H (2015) Variable number of tandem repeat polymorphisms in DGAT1 gene of buffaloes (*Bubalus bubalis*) is associated with milk constituents. *Journal of Dairy Science* **93**, 492–495.

Cases S, Smith SJ, Zheng YW, Myers HM, Lear SR, Sande E, Novak S, Collins C, Welch CB, Lusis AJ, Erickson SK and Farese RV (1998) Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 13018–13023.

Freitas AC, de Camargo GM, Aspilcueta-Borquis RR, Stafuzza NB, Venturini GC, Tanamati F, Hurtado-Lugo NA, Barros CC and Tonhati H (2016*a*) Polymorphism in the A2M gene associated with high-quality milk in Murrah buffaloes (*Bubalus bubalis*). *Genetics and Molecular Research: Genetics Molecular Research* **15**, 1–7.

Freitas AC, de Camargo GM, Stafuzza NB, Aspilcueta-Borquis RR, Venturini GC, Dias MM, Cardoso DF and Tonhati H (2016*b*) Genetic association between SNPs in the DGAT1 gene and milk production traits in Murrah buffaloes. *Tropical Animal Health and Production* **48**, 1421–1426.

Gautier M, Capitan A, Fritz S, Eggen A, Boichard D and Druet T (2007) Characterization of the DGAT1 K232A and variable number of tandem repeat polymorphisms in French dairy cattle. *Journal of Dairy Science* **90**, 2980–2988.

Li S, Wang L, He X, Xie Y and Zhang Z (2015) Identification and analysis of the promoter region of the STGC3 gene. *Archives of Medical Science* **11**, 1095–1100.

Ménard O, Ahmad S, Rousseau F, Briard-Bion V, Gaucheron F and Lopez C (2010) Buffalo vs. cow milk fat globules: size distribution, zeta-potential, compositions in total fatty acids and in polar lipids from the milk fat globule membrane. *Food Chemistry* **120**, 544–551.

Parikh RC, Patel JV, Patel AB, Patel KS, Patel TB, Patil RC, Kansara JD, Jakhesara SJ and Rank DN (2016) DGAT1 Gene polymorphisms and its association with milk production traits in Mehsana buffalo (*Bubalus bubalis*). *Buffalo Bulletin* **2**, 237–246.

Sandelin A, Carninci P, Lenhard B, Ponjavic J, Hayashizaki Y and Hume DA (2007) Mammalian RNA polymerase II core promoters: insights from genome-wide studies. *Nature Reviews Genetics* **8**, 424–436.

Silva CS, Silva Filho E, Matos AS, Schierholt AS, Costa MR, Marques LC, Costa JS, Sales RL, Figueiró MR and Marques JR (2016) Polymorphisms in the DGAT1 gene in buffaloes (*Bubalus bubalis*) in the Amazon. *Genetics and Molecular Research* **15**, 3.

White SL, Bertrand JA, Wade MR, Washburn SP, Green JT and Jenkins TC (2001) Comparison of fatty acid content of milk from Jersey and Holstein cows consuming pasture or a total mixed ration. *Journal of Dairy Science* **84**, 2295–2301.

Winter A, Krämer W, Werner FA, Kollers S, Kata S, Durstewitz G, Buitkamp J, Womack JE, Thaller G and Fries R (2002) Association of a lysine-232/alanine polymorphism in a bovine gene encoding acyl-CoA: diacylglycerol acyltransferase (DGAT1) with variation at a quantitative trait locus for milk fat content. *Proceedings of the National Academy of Sciences* **9**, 9300–9305.

Yuan J, Zhou J, Deng X, Hu X and Li N (2007) Molecular cloning and single nucleotide polymorphism detection of buffalo DGAT1 gene. *Biochemical Genetics* **45**, 611–621.