Trop Anim Health Prod (2016) 48:1343–1349
DOI 10.1007/s11250-016-1092-8

REGULAR ARTICLES

A novel selection signature in stearoyl-coenzyme A desaturase (*SCD*) gene for enhanced milk fat content in *Bubalus bubalis*

J. Maryam[1] · **M. E. Babar**[2] · **Zhang Bao**[3] · **A. Nadeem**[1]

Received: 22 January 2016 / Accepted: 8 June 2016 / Published online: 16 June 2016

Abstract Modern molecular interventions are dynamic gears for breeding animals with superior genetic make-up. These scientific efforts lead us toward sustainable dairy herds with improved milk production in terms of yield and quality. Many of candidate genes have been dissected at molecular level, and suitable genetic markers have been identified in cattle, but this work has not been validated in buffaloes so far. Stearoyl-coenzyme A desaturase (SCD) has been a potential candidate gene for fat content of milk. Genomic analysis of SCD revealed a total of six variations that were identified through DNA sequencing of animals with lower and higher butter fat %age. After statistical analysis, genotype AB of p.K158I could be associated (*P* value <0.0001) with higher milk fat %age (10.5 ± 0.5464). This SNP was validated on larger data set by cleaved amplified polymorphic sequences (CAPS) by using DdeI. To scrutinize the functional consequences of p.K158I, 3D protein structure of SCD was predicted by homology modeling and this variation was found located in the vicinity of functional domain and a part of transmembrane helix of this membrane integrated protein. This is a first report toward genetic screening of SCD gene at molecular level in buffalo. This report illustrates the implication of SCD gene and in particular p.K158I variation, in imparting its effect on milk fat %age, which can be targeted in selection of superior dairy buffaloes.

Keyword SCD gene · Dairy buffalo · Fat %age · Variation · CAPS · 3D protein structure

✉ A. Nadeem
asifnadeem@uvas.edu.pk

[1] Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Sheikh Abdul Qadir Jillani Raod, Lahore 54000, Pakistan

[2] Virtual University of Pakistan, Lahore, Pakistan

[3] Xi'an Jiaotong University, Xi'an 710061, China

Introduction

Milk production is the area of focus for modern geneticists in recent years. Due to ever increasing demand of milk as nutritive dietary component, there are apprehensions regarding existing production potentials of dairy animal (both in yield and quality). So, recent scientific efforts are targeting genetic basis of milk production trait to identify genetic loci imparting significant role to enhance future production of dairy herds. Many of the candidate genes have been tested to find their role in determining milk production of animals (Jiang et al. 2010; Mullis 2007; Svennersten-Sjaunja and Olsson 2005; Li et al. 1990; Goddard and Hayes 2009; Grisart et al. 2002; Khatib et al. 2007, 2008). But, majority of identified markers have not been found informative in buffaloes, which are major dairy contributors in Asian regions and many other parts of world as well. So, there is need to work on genomic exploration of river buffalo for identification of novel selection signatures.

Stearoyl-coenzyme A desaturase (SCD) has been determined as potential gene effecting milk fatty acids profile

Table 1 Polymorphic loci in SCD gene and HWE test ($P < 0.05$)

Location	SNP ID	Position	Change in nucleotide	Amino acid substitution	Chi test ($P < 0.05$)	HWE
Exon-II	p.2648	2648	G → A	–	0.007	S
	p.S80F	2779	C → T	S-F	0.254	NS
Intron	p.6596	6596	G → C	–	0.014	S
Exon-IV	p.K158I	8453	A → T	K-I	2.176	NS
	p.8505	8505	G → A	–	0.258	NS
Exon-V	p.D239G	10153	A → G	D-G	0.17	NS

S significant, *NS* nonsignificant

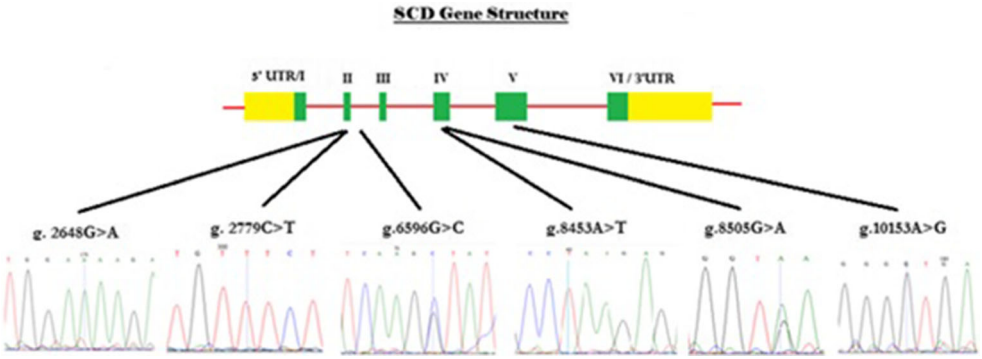

Fig. 1 SCD gene structure and position of polymorphisms obeying HWE (*green boxes* are exons and *yellow boxes* are UTR) (color figure online)

(Gautier et al. 2006; Bauman et al. 2006; Soyeurt et al. 2006). SCD is a multifunctional complex enzyme important in the cellular biosynthesis of fatty acids (Ntambi and Miyazaki 2004). The SCD locus has been mapped on Bos taurus autosome (BTA)26 (Campbell et al. 2001). Some of significant associations have been reported in different cattle breeds (Ntambi and Miyazaki 2004; Campbell et al. 2001; Kgwatala et al. 2007; Taniguchi et al. 2004). The aforementioned results illustrate a probable significance of the SCD locus in gene-assisted selection programs for the genetic improvement of milk fat quality in dairy buffaloes as well.

In this context, present research was intended to screen the SCD gene in river buffalo of Pakistan. All six exons of the gene were sequenced and six variations were reported (Maryam et al. 2013). After statistical analysis, six variations were found nonsignificant potential loci ($P > 0.05$) for association studies. p.K158I was selected for obeying Hardy–Weinberg equilibrium (HWE) with significance ($2.176 > 0.05$). This region was genotyped by restriction digestion on larger buffalo population and was further subjected to association analysis. It was concluded that genotype AB was found associated with milk fat content in buffaloes.

This region was further analyzed for 3D protein structure to find functional consequences of the polymorphism. It was determined that p.K158I was located inside of the transmembrane region of SCD protein and in the vicinity of a functional domain. As this was a hydrophilic to hydrophobic substitution, this might have a role in increase of milk fat content in bovines.

Materials and methods

Phenotypic data set

River buffalo breed Nili-Ravi was selected as experimental animal because of its high milk butter fat potential (on an average 8 %). Animals were selected from Buffalo Research Institute (BRI), Pattoki, Pakistan, in their first month of second lactation. Fat %age (FP) of individual animal was taken from Production Record Data Sets available at farm. A total of 69 animals were selected for initial sequencing. On the basis of fat %age record, animals were categorized into two groups (group 1: FP > 8.0, group 2: FP < 8.0) and blood sampling was conducted.

Table 2 Reaction recipe for restriction digestion

Amplified template	10 μl
Restriction buffer	2 μl
Restriction enzyme	0.7 μl
H_2O	17.3 μl

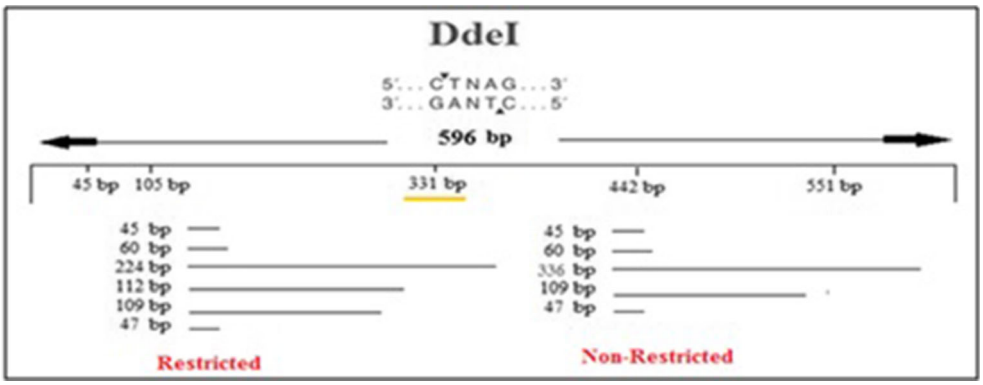

Fig. 2 Restriction map of SCD-22. SCD-22 illustrated significant association with milk fat %age. Restriction was done with DdeI targeting CT

Genomic DNA amplification and sequencing

Genomic DNA was extracted by using organic DNA extraction protocol reported by Maryam et al. (2012). Exonic region of SCD gene was amplified by using specific sets of primers reported by Alim et al. (2012). For initial study of gene, 69 animals ($n = 34$ from each group) were amplified by using 0.5 μl of each primer, 2.5 μl 109 PCR buffer, 2.5 mM each of dNTP, and 1 U of Taq DNA polymerase. The PCR protocol was 5 min at 94 °C for initial denaturing followed by 34 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min for all the primers. Then, each PCR product was sequenced by Sanger's chain termination method using the ABI3730XL (Applied Biosystems, Foster City, CA). Sequences from both groups were aligned on BLAST resource of NCBI and a total of six variations were identified (Table 1).

Statistical analysis

POPGENE (http://www.ualberta.ca/*fyeh/) version 1.32 was used to determine Hardy–Weinberg equilibrium (HWE) of each variation. Some of the variations could not be analyzed because of monomorphic nature in population. Some other was found significantly deviated from HWE and so could not be useful in association analysis. p.K158I was selected for further screening on larger data set (Fig. 1) and was further tested for probable association with milk fat % age.

CAPS

p.K158I was genotyped at larger data set to validate the association in animals from different livestock farms. Almost 346 more animals of Nili-Ravi buffaloes (Livestock Experimental Station, Bahadur nagar, Okara and Buffalo Colony, Karachi) were genotyped by cleaved amplified polymorphic sequences (CAPS). For this purpose, DdeI enzyme was selected and restriction was performed on PCR-amplified fragment of the SCD gene. Recipe for restriction digestion is given in Table 2. Restriction map was studied to determine the genotypic pictorial of the p.K158I (Fig. 2 and 3). After this screening, association analysis and genotypic frequencies were tested as mentioned in Tables 3, 4, and 5.

Association analysis

Association was performed by one-way ANOVA by calculating (mean ± SE). p.S80F was having weaker association with probability of 0.049444, while p.K158I was found to have stronger association with $P \leq 0.0001$. Genotypic and allelic frequencies for these six loci were also calculated by POPGENE32 (Tables 3 and 4). It was found that genotype AB at p.K158I was strongly associated with high milk fat %age (10.5 ± 0.5464) (Table 5).

3D protein model prediction and analysis

Three-dimensional protein model was predicted by using PyMOL (www.pymol.org) and Phyre2 (www.sbg.bio.ic.ac.uk/phyre/html/). As no previous information could be retrieved for structural attributes of SCD protein, this model was further analyzed for prediction of probable

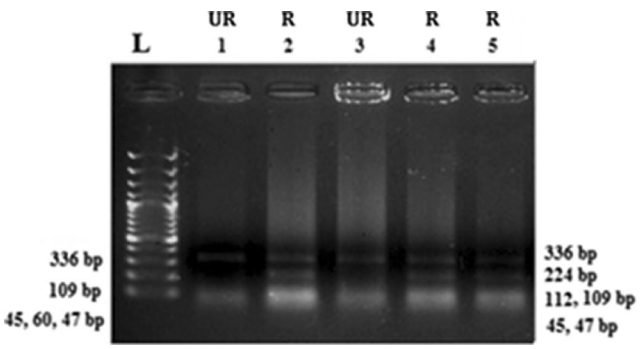

Fig. 3 Electrophoretic pictorial of screening of SCD-22 in river buffalo. *UR* unrestricted when mutation is absent, *R* restricted when mutation is present

Table 3 Genotypic frequencies of six SNPs of SCD gene in river buffalo

			
p.2648	0.25	0.34	0.41
p.S80F	0.47	0.24	0.29
p.6596	0.12	0.47	0.41
p.K158I	0.18	0.78	0.04
p.8505	0.28	0.54	0.18
p.D239G	0.70	0.12	0.18

Table 4 Genotypic frequencies of six SNPs of SCD gene in river buffalo

	p.2648	p.S80F	p.6596	p.K158I	p.8505	p.D239G
A allele	0.6585	0.6988	0.6029	0.3659	0.9759	0.7711
B allele	0.3415	0.3012	0.3971	0.6341	0.0241	0.2289

functional domains, signal peptides, secondary structures, and transmembrane helix (Figs. 4 and 5).

Results

SCD gene was analyzed in buffaloes to identify genetic variations. A total of six variations were identified in exonic and some of intronic region of SCD. Exon-IV was found to have major proportion of all variations (31 %). This information is given in Table 1. After HWE testing (χ^2), SNPs obeying the Hardy–Weinberg law (Table 1) were further tested for association with fat content by calculating (mean ± SE). p.K158I was genotyped by CAPS on larger animal population and was found to have stronger association with higher fat content. Restriction mapping (Figs. 2 and 3) was done to validate the association probability at larger data set association of p.K158I illustrated that buffaloes with genotype AB were showing the maximum FP ($P \leq 0.0001$) (Table 5). So, it was concluded that buffaloes with heterozygous genotype AG were depicting high FP than other genotypes.

Finally, 3D protein structure of SCD protein (359 amino acid residues) was predicted on the basis of sequence homology (Figs. 4 and 5). Predicted model for this protein was

Table 5 Single marker association by one-way ANOVA (mean ± SE)

Genetic variations	AA (mean ± SE)	AB (mean ± SE)	BB (mean ± SE)	*P* value (0.05)
p.2648	$n = 139$	$n = 76$	$n = 131$	0.154917
	5.46 ± 0.6046^a	6.1 ± 1.0985^a	4.12 ± 0.2709^a	
p.S80F	$n = 43$	$n = 211$	$n = 92$	0.049444
	6.14 ± 0.7646^a	9.0 ± 1.0649^b	6.2 ± 0.4308^a	
p.6596	$n = 127$	$n = 73$	$n = 146$	0.990059
	6.26 ± 1.0107^a	6.1 ± 1.0985^a	6.12 ± 1.4538^a	
p.K158I	$n = 61$	$n = 192$	$n = 93$	<0.0001
	4.34 ± 0.4069^a	10.5 ± 0.5464^b	5.5 ± 0.2187^a	
p.8505	$n = 121$	$n = 211$	$n = 14$	0.096742
	6.06 ± 0.8588^a	9.35 ± 0.1893^a	6.12 ± 1.4538^a	
p.D239G	$n = 83$	$n = 139$	$n = 124$	0.581376
	6.06 ± 0.8588^a	7.85 ± 1.5283^a	6.12 ± 1.4538^a	

P value refers to the results of association analysis between each SNP and milk Fat %age. Individual sequencing data has been used ($n = 593$). Means within a row with different superscripts differ ($P < 0.05$)

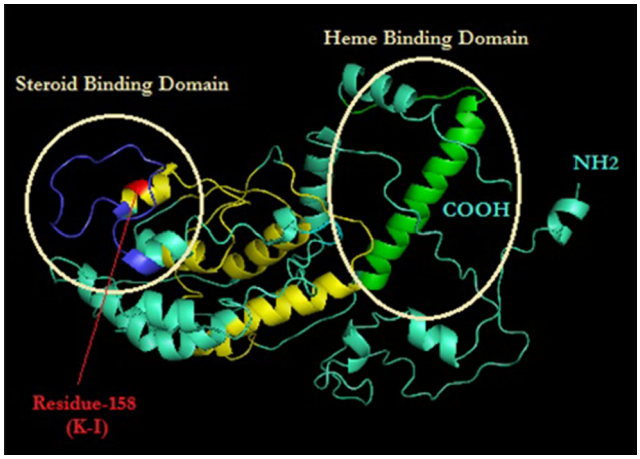

Fig. 4 SCD protein model prediction by homology modeling: *Yellow color* illustrates transmembrane helices; *green color* is denoting Heme Binding Domain; *blue color* is indicating steroid binding domain; and *red color* is locating amino acid substitution from *K* to *I* (color figure online)

carrying 58 % alpha helices and 2 % beta sheets (Fig. 6). p.K158I was found as a part of transmembrane helix at position no. 158 (Table 6) and in the vicinity of steroid-binding domain. As this was part of functional protein and amino acid substitution was also hydrophilic to hydrophobic exchange, functional consequences of p.K158I could be predicted.

Discussion

A novel selection signature was identified in SCD gene that was found to be associated with higher milk fat content. A study was carried out by Macciotta et al. (2008), who claimed that cows with homozygous genotype CC at the SCD locus (g.10329C/T) producing more milk (about 2 kg/day) and protein (about 0.07 kg/day) compared with TT cows; heterozygous cows were in an intermediate position, but our results deviate from reported literature. The results of present research are contrasting than Macciotta but are in accordance with Alim et al. (2012) who also reported association of heterozygous genotypes with different milk production traits. B allele was found to have more frequency than A. As no efforts in terms of selective breeding have been initiated yet, so this might be due to natural genetic drift and provides a potential for future marker-assisted selection programs to enhance fat content of milk. Another study by Conte et al. (2006), on Italian brown cattle reported a significant association between SCD1 genotypes and milk FA C14:1 cis-9 and DI 14 (P B 0.01). In particular, the CC genotype was associated with 18.3 and 20.6 % more C14:1 cis-9 and DI 14, respectively, compared with the TT genotype. These apparently inconsistent results might be justified as experimental breed of this research was Nili-Ravi, which is a cross of Nili and Ravi buffalo breeds of Pakistan. This crossing although is being done over the past 100 years or even more, both breeds still hold a distinctive place among best dairy buffalo breeds in the world. So, cross-breeding of these animals resulted in pooling of better genotypes controlling different production traits. Now, because of its production potentials, better adaptability in the hot and humid climate of Pakistan, and better immunity, Nili-Ravi has been declared Black Gold of Asia.

Finally, 3D protein structure prediction was performed. Very limited literature information regarding protein structural

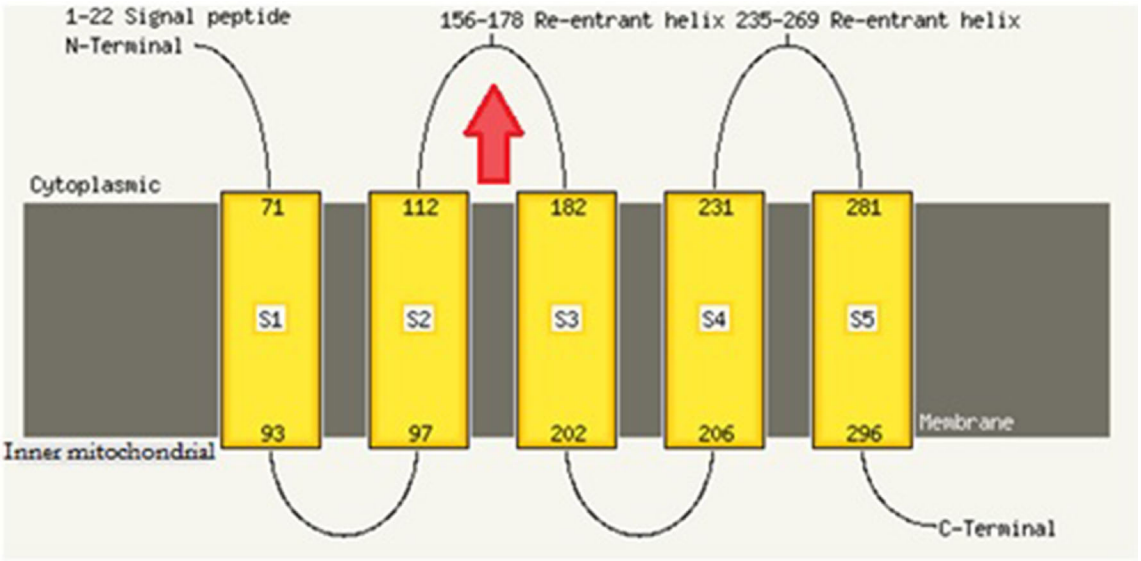

Fig. 5 Transmembrane helix illustrated potential site for SCD-22. *Red arrow* indicates location of 158th residue, which is exposed to cytoplasm (color figure online)

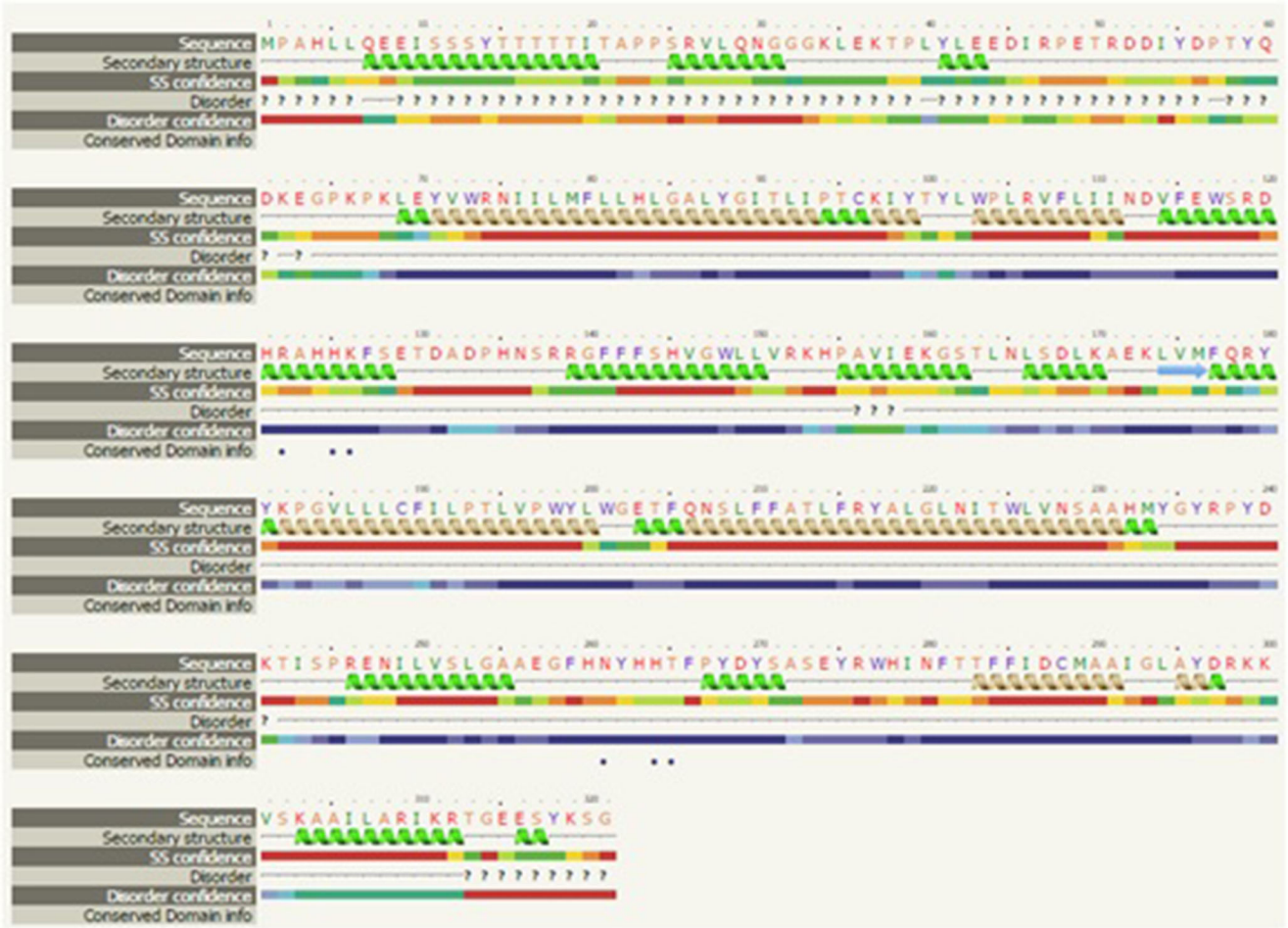

Fig. 6 Secondary structure prediction of SCD protein. Alpha helix = 62 %, beta strands = 2 %, and TM helix = 32 % (Phyre2 software)

attributes could be found. Stearoyl-CoA has no known paralogs. It, however, does have 19.4 % homologs in mammals and 20.8 % homologs in bacteria, so it is likely that these organisms have similar oxidoreductases that catalyze desaturation reactions as does this enzyme (Xiaoyun et al. 2013). The stearoyl-CoA desaturase system in animals is anchored in the endoplasmic reticulum membrane via four hydrophobic transmembrane domains with the active center (and the N- and C-termini) exposed to the cytosol (Buist 2004). Abovementioned literature reported transmembrane nature of SCD protein which is mitochondrial and is major enzyme in fatty acid synthesis. Two of the domains have been reported previously heme/steroid-binding domain and fatty acid desaturase domain (Buist 2004). p.K158I was found in the vicinity of steroid-binding domain, which is functionally active region for fatty acid synthesis. Observing, the abovementioned results following assumptions were made: This variation is switching of polarity to nonpolarity and hydrophilicity to hydrophobicity and so might have better transport across mitochondrial membrane and enhanced fatty acid production, or presence of this substitution in the vicinity of steroid-binding domain might affect protein functionality. These assumptions need further confirmations by advanced proteomic approaches. But, so far, this is first report regarding variation at amino acid level which is affecting fat content in the milk of bovines. These results suggest that SCD is a candidate gene that influences milk production traits and provides potentially useful information for dairy breeding programs.

Table 6 Amino acid substitution at p.K158I found in potential transmembrane region of SCD protein

Variation	Residual position	Substitution	Group	Effect
K-I	158	Lys-Iso	Polar to nonpolar	Hydrophilic to hydrophobic

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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