

# Two single nucleotide polymorphisms within bovine butyrophilin gene (*BTN/HaeIII* and *BTN/SchI*) and their association with milk performance traits in Jersey cattle

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## Abstract

Associations between two polymorphisms localized in the seventh exon of the butyrophilin gene (*BTN/HaeIII* and *BTN/SchI*) and milk production traits of Jersey cattle were analysed. A total of 171 cows were included in the study. PCR-RFLP method was used. In both polymorphisms three genotypes were identified. Statistically significant differences between polymorphic variants and milk production traits of Jersey cows were observed. Those differences concerned the percentage fat content in milk in the case of both polymorphisms and additionally the yield of milk and protein in the case of *BTN/HaeIII* polymorphism. The influence of the combined genotypes (*BTN/HaeIII/SchI*) on the examined traits was also analysed. *GG<sup>HaeIII</sup>/AG<sup>SchI</sup>* combined genotype was characterized by a significantly lower percentage fat content in comparison to other combinations. Moreover, incomplete linkage between the studied polymorphic sites was observed, despite relatively small distance between them.

**Keywords:** butyrophilin gene, PCR-RFLP, dairy cattle, milk production traits, Jersey

## Zusammenfassung

### Zwei einzelne Nukleotidpolymorphismen innerhalb des Butyrophilin-Gens (*BTN/HaeIII* und *BTN/SchI*) und ihr Zusammenhang mit den Milchproduktionsmerkmalen der Jersey-Rinder

Zusammenhänge zwischen den im siebten Exon des Butyrophilin-Gens lokalisierten Polymorphismen (*BTN/HaeIII* und *BTN/SchI*) und den Milchleistungsmerkmalen von Jersey-Rindern wurden analysiert. Die Untersuchungen umfassten 171 Kühe. Es wurde die PCR-RFLP-Methode angewandt. Signifikante Unterschiede zwischen den *BTN/HaeIII*- und *BTN/SchI*-Polymorphismen und den Milchleistungsmerkmalen wurden festgestellt. Diese Unterschiede betreffen den prozentualen Fettgehalt bei beiden Polymorphismen, die Milchleistung sowie den Eiweißgehalt im Falle des Polymorphismus *BTN/HaeIII*. Die Polymorphismen wurden im System der kombinierten Genotypen analysiert. Der kombinierte Genotyp *GG<sup>HaeIII</sup>/AG<sup>SchI</sup>* war charakterisiert durch einen signifikant niedrigeren Prozentgehalt an Fett im Vergleich zu anderen Kombinationen. Zusätzlich wurde keine Konjugation zwischen den analysierten Polymorphismen gefunden, obwohl eine relativ kurze Entfernung zwischen beiden Polymorphismen besteht.

**Schlüsselwörter:** Butyrophilin-Gen, PCR-RFLP, Milchrind, Milchproduktionsmerkmale, Jersey-Rind

## Introduction

The butyrophilin protein was identified as a main component of the milk fat globule membrane (MFGM), which is important in secreting and stabilizing of milk-fat droplets in milk (FRANKE *et al.* 1981). Its existence was discovered and characterized in milk of many species, including human. Butyrophilin comprises 34-43 % of the total MFGM proteins in the milk of Holstein-friesian cows and approximately 20 % in the milk of Jersey cows (MATHER 2000). Bovine butyrophilin consists of 526 amino acids (~56 kDa), and the first 26 of them constitute a signal peptide (JACK and MATHER 1990). Butyrophilin protein exhibits a domain character of build and is a type I transmembrane glycoprotein (BANGHART *et al.* 1998) strongly connected with the cell membrane (JACK and MATHER 1990, MATHER and JACK 1993).

The function that butyrophilin plays in a living organism remains unknown. Taking into consideration that butyrophilin expression is strictly limited only to the apical surfaces of epithelial secretory cells of mammary gland and its presence in structures surrounding milk-fat droplets in milk, it seems that butyrophilin protein is essential for the proper course of fat secretion process into milk. What is characteristic, butyrophilin is highly expressed only during the lactation and last period of pregnancy (FRANKE *et al.* 1981, MATHER and JACK 1993).

The bovine butyrophilin gene (*BTN*) was mapped to the long arm of chromosome 23 [23q21-q23] (BRUNNER *et al.* 1996, TAYLOR *et al.* 1996). It comprises seven exons and six introns (DAVEY *et al.* 1997). The gene structure organization corresponds to the distribution of functional domains of coding protein (DAVEY *et al.* 1997, OGG *et al.* 2004). Additionally, 5'UTR and 3'UTR are present in the butyrophilin gene structure. In 5'UTR region a short intronic sequence was found, which shows that, in the case of bovine butyrophilin gene, this region is coded by two separate exons (KOMISAREK and DORYNEK 2002, SEYFERT and LUTHEN 1998), opposed to other species, in which 5'UTR is continuous (SEYFERT and LUTHEN 1998). The butyrophilin gene promoter is an example of TATA-less promoters. *In silico* analysis of the bovine butyrophilin promoter region showed a number of potential transcription factor binding sites (DAVEY *et al.* 1997). Butyrophilin gene expression is strictly regulated at the transcription level by lactogenic hormones (BANGHART *et al.* 1998).

Within the bovine butyrophilin gene, a number of polymorphic sites were identified, both in coding (exons) and noncoding (introns) regions (HUSAINI *et al.* 1999, SEYFERT and LUTHEN 1998, TAYLOR *et al.* 1996, ZEGEYE *et al.* 1999). A list of all discovered polymorphic sites, together with their location and possible influence on amino acid sequence, is showed in Table 1. Due to the function of the protein itself and the localization of the butyrophilin gene in the close neighborhood of the genes associated with the major histocompatibility complex (MHC) and several potential markers connected with production traits (ASHWELL *et al.* 1999), the butyrophilin gene was initially classified to the group of QTL candidate genes. The attempt to characterize the influence of particular polymorphic variants of this gene on production traits i.e. content of individual milk-components in milk (KOMISAREK and DORYNEK 2002, KOMISAREK *et al.* 2006b) or somatic cell count (KOMISAREK *et al.* 2006a) was made.

The aim of the study was to determine the frequency of occurrence of particular genotypes and alleles of *BTN* gene (polymorphic sites in positions 6804 and 6845) and to analyze of the relationship between examined polymorphic variants and milk performance traits in Jersey cows.

Table 1

Summary of a point mutations within the bovine butyrophilin (bBTN) gene

*Zusammensetzung der Punktmutationen innerhalb der Butyrophilingene (BTN)*

	Nucleotide position*	Localisation of mutation	Type of mutation	Influence on amino acid sequence	Reference
1.	243	5'UTR	T→C	–	(HUSAINI <i>et al.</i> 1999)
2.	1849	Exon 1	T→A	Phe→Tyr	(HUSAINI <i>et al.</i> 1999)
3.	1939	Intron A	A→C	–	(ZEGEYE <i>et al.</i> 1999)
4.	2074	Intron A	A→T	–	(ZEGEYE <i>et al.</i> 1999)
5.	2076	Intron A	A→G	–	(ZEGEYE <i>et al.</i> 1999)
6.	2281	Exon 2	C→A	Pro→Gln	(SEYFERT and LUTHEN, 1998)
7.	3645	Intron B	G→T	–	(HUSAINI <i>et al.</i> 1999)
8.	3713	Intron B	G→A	–	(HUSAINI <i>et al.</i> 1999)
9.	4226	Exon 3	A→T	Glu→Asp	(HUSAINI <i>et al.</i> 1999)
10.	5495	Intron D	G→A	–	(ZEGEYE <i>et al.</i> 1999)
11.	6658	Exon 7	C→G	Missens	(HUSAINI <i>et al.</i> 1999)
12.	6673	Exon 7	C→T	Missens	(HUSAINI <i>et al.</i> 1999)
13.	6804	Exon 7	A→G	Lys→Arg	(TAYLOR <i>et al.</i> 1996)
14.	6845	Exon 7	G→A	Val→Iso	(HUSAINI <i>et al.</i> 1999)
15.	6958	Exon 7	A→C	Leu→Pro	(HUSAINI <i>et al.</i> 1999)

\* Nucleotide position of mutation according to complete BTN gene sequence (GenBank Acc. No Z93 323)

## Material and methods

The study involved 171 Jersey cows from the Siedlec herd (Central region of Poland) derived from 101 sires and 160 dams. The biological material for the study was peripheral blood, taken from external jugular vein and stored in test tubes containing anticoagulant (K<sub>3</sub>EDTA). DNA isolation was conducted using MasterPure™ DNA Purification Kit (EPICENTRE® Biotechnologies). Genotypes of animals were determined using PCR-RFLP method.

The PCR reaction was performed in GeneAmp 2400 (Perkin Elmer) thermocycler. The reaction mixture (20 µl) contained: polymerase buffer, nucleotide mix (0.2 mM), MgCl<sub>2</sub> (2.5 mM), F and R primers (1 µM), ~60 ng of genomic DNA, *Taq* polymerase (0.5 unit) (Fermentas) and deionized water (up to 20 µl). The following temperature profile was used: initial denaturation of templates for 5 min at a temperature of 94 °C, the next 35 cycles were repeated; denaturation at 94 °C/60 s, annealing at 64 °C/60 s, extension at 72 °C/90 s and in the end, a final PCR product extension at a temperature of 72 °C for 5 min.

In the amplification reaction, primers proposed by Komisarek and Dorynek (KOMISAREK and DORYNEK 2002) were used, with the modification of reverse (R) primer, which allowed for simultaneous detection of two polymorphic sites:

F: 5'-TGG AGC TCT ATG GAA ATG GG-3'

R: 5'-GGA ATA GGC CCT TCC TAA TC-3'

A 568 bp fragment of *BTN* gene was amplified. Polymorphic sites were identified using restriction enzymes; the A→G substitution in position 6 804 using *HaeIII* enzyme (KOMISAREK and DORYNEK 2002), and the substitution G→A in position 6 845 using *SchI* enzyme. The restriction pattern for *SchI* enzyme was determined using NEBcutter (VINCZE *et al.* 2003). For both enzymes (Fermentas), the digestion was conducted at 37 °C (minimum 3 h or overnight) using 4 units of each restriction enzyme. Restriction fragments were separated in agarose gels; in the case of *HaeIII* digestion separation was conducted in 2 % gel (BASICA GQT, Prona), at 120V, in 1X TBE buffer, and in the case of *SchI* digestion, 3 % agarose gel was used (70V, 1X TBE). Gels were stained with ethidium bromide (1.0 µM) (AppliChem), and the results were analysed in UV light (312 nm).

Data on the milk performance of cows was obtained from the farm documentation. Analysis of the level of traits associated with milk performance (overall milk, protein and fat yields as well as fat and protein contents) was conducted according to polymorphic variants of butyrophilin gene using the linear model:

$$Y_{ijkl} = \mu + G_i + s_j + d_k + L_l + \alpha (x - x_m) + \beta (dm - dm_m) + e_{ijkl} \quad (1)$$

where  $Y_{ijkl}$  is the analysed trait,  $\mu$  is the overall mean,  $G_i$  is the fixed effect of the analysed genotypes from each polymorphism,  $s_j$  is the random effect of a sire,  $d_k$  is the random effect of a dam,  $L_l$  is the fixed effect of the number of lactation,  $\alpha$  is the regression coefficient for the cow calving age,  $x$  is the calving age of a cow,  $x_m$  is the mean calving age of the analysed cows,  $\beta$  is the regression coefficient for the number of days in milk,  $dm$  is the days in milk of a cow,  $dm_m$  is the mean number of days in milk of cows and  $e_{ijkl}$  is the random error.

Analysis were conducted using Statistica® 8.0 package.

## Results

As a result of conducted PCR-RFLP analysis three genotypes were identified (AA, AG i GG) for both restriction enzymes (*HaeIII* and *SchI*) Figure 2. The frequencies of alleles and genotypes for the analysed population are shown in Table 2.

Table 2  
Frequencies of alleles and genotypes of *BTN/HaeIII* and *BTN/SchI* in studied population of cows  
*Allel- und Genotypfrequenzen BTN/HaeIII i BTN/SchI in der untersuchten Population*

Polymorphism	Genotypes			Alleles	
<i>BTN/HaeIII</i>	AA	AG	GG	A	G
	0.480	0.421	0.099	0.690	0.310
	(n=82)	(n=72)	(n=17)		
<i>BTN/SchI</i>	AA	AG	GG	A	G
	0.205	0.357	0.438	0.383	0.617
	(n=35)	(n=61)	(n=75)		

The effect of each genotype (*BTN/HaeIII* and *BTN/SchI*) on milk production traits was analysed in this study. The results are presented in Table 3.

Owing to a lack of full linkage between the studied polymorphic sites, except for isolated genotypes, the influence of the combined genotypes (*BTN/HaeIII/Schl*) on the examined phenotype traits was also analysed (Table 4).

Table 3

Means and standard deviations (SD) of milk production traits in Jersey cows carrying different *BTN* genotypes  
*Durchschnittliche Standardabweichungen von Milchproduktionsmerkmalen bei Jersey-Rindern in Abhängigkeit vom Genotyp*

Genotype	Milk, kg		Fat, kg		Fat, %		Protein, kg		Protein, %	
<i>BTN/HaeIII</i>	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
AA	4508 <sup>A</sup>	816	252.27	47.96	5.62 <sup>a</sup>	0.55	174.84 <sup>A</sup>	31.64	3.88	0.24 <sup>AG</sup>
	4559 <sup>B</sup>	827	253.13	45.72	5.61	0.52	178.25	30.60	3.92	0.23
GG	4853 <sup>AB</sup>	789	265.93	48.42	5.47 <sup>a</sup>	0.36	186.35 <sup>A</sup>	31.10	3.84	0.22
<i>BTN/Schl</i>	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
AA	4447	815	247.06	46.74	5.57 <sup>a</sup>	0.51	172.78	31.25	3.89	0.23
AG	4571	789	254.86	46.38	5.62 <sup>a</sup>	0.55	179.71	31.08	3.92	0.23
GG	4688	855	260.87	47.67	5.61	0.51	180.02	31.09	3.87	0.25

Means in columns with the same letter differ significantly, capitals  $P \leq 0.01$ , small letters  $P \leq 0.05$ .

Table 4

Means and standard deviations (SD) of milk production traits in Jersey cows carrying different *BTN* combined genotypes

*Durchschnittliche Standardabweichungen von Milchproduktionsmerkmalen bei Jersey-Rindern in Abhängigkeit von den kombinierten Genotypen*

Combined genotype ( <i>HaeIII/Schl</i> )	Milk, kg	Protein, kg	Protein, %	Fat, kg	Fat, %
AA/AA	4405	171.08	3.89	246.49	5.61 <sup>B</sup>
(n=28)	(859)	(33.11)	(0.22)	(49.42)	(0.52)
AA/AG	4487	169.87	3.78 <sup>ab</sup>	242.80	5.44 <sup>adg</sup>
(n=5)	(606)	(25.81)	(0.22)	(43.10)	(0.86)
AA/GG	4669	181.54	3.90	262.93	5.66 <sup>abcA</sup>
(n=49)	(767)	(29.58)	(0.28)	(45.23)	(0.53)
AG/AA	4620	180.33	3.92 <sup>b</sup>	250.10	5.43 <sup>beh</sup>
(n=11)	(680)	(24.10)	(0.30)	(38.00)	(0.55)
AG/AG	4570	180.55	3.94 <sup>a</sup>	256.27	5.65 <sup>ghid</sup>
(n=51)	(808)	(31.09)	(0.22)	(46.87)	(0.51)
AG/GG	4458	166.07	3.83	240.93	5.57 <sup>C</sup>
(n=10)	(1015)	(30.72)	(0.19)	(44.79)	(0.55)
GG/AA	4530	175.23	3.87	247.08	5.44 <sup>cfi</sup>
(n=9)	(587)	(23.66)	(0.23)	(37.11)	(0.35)
GG/AG	4891	189.50	3.84	254.75	5.21 <sup>defBCDE</sup>
(n=2)	(895)	(48.37)	(0.30)	(46.37)	(0.07)
GG/GG	5030	192.09	3.82	278.52	5.54 <sup>E</sup>
(n=6)	(843)	(31.31)	(0.21)	(52.08)	(0.38)

Means in columns with the same letter differ significantly, capitals  $P \leq 0.01$ , small letters  $P \leq 0.05$ .

## Discussion

It is supposed that, in milk-fat secretion process, butyrophilin interacts with other proteins that are the part of milk-fat globule membranes (MFGM) i.a. with xanthin oxidase (XO) and adipophilin (ADPH) Figure 1 (MATHER and KEENAN 1998, McMANAMAN *et al.* 2007). Interaction of BTN and XO complex with adipophilin and probably other proteins present on the surface of intracellular lipid droplets could be involved in moving the lipid droplets closer to the apical part of cell membrane (HEID and KEENAN 2005).

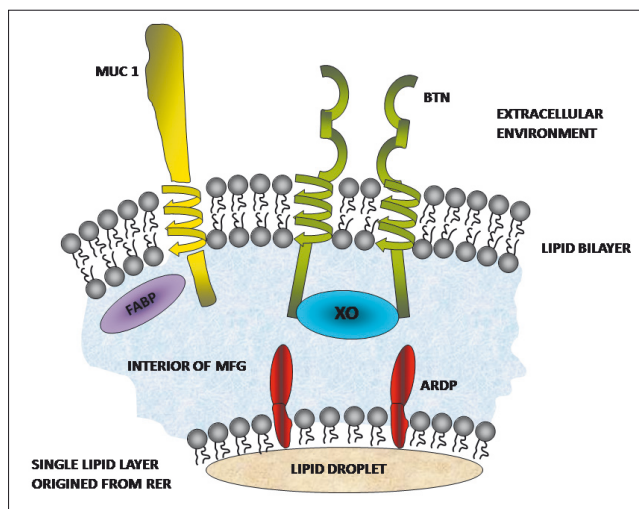


Figure 1  
Putative arrangement of selected proteins present in the MFGM structure. MUC 1 mucin, XO xanthin oxidase, BTN butyrophilin, ADPH adipophilin, FABP fatty acids binding protein. (Prepared on the basis of: MATHER and JACK 1993, MATHER and KEENAN 1998, McMANAMAN *et al.* 2007)  
*Mutmaßliche Anordnung von ausgewählten Proteinen in der MFGM-Struktur.*

An importance of butyrophilin in milk-fat secretion process was confirmed by experiments carried out on *Btn* gene knock-out mice (*Btn*<sup>-/-</sup>), in which lipids were accumulated in mammary epithelial cells, but were not released at all from the cell (OGG *et al.* 2004). Interesting results have been presented in studies of the structure of milk-fat globule membranes, conducted using freeze-fracture technique, which point at dominant function of butyrophilin in controlling the process of milk-fat secretion (ROBENEK *et al.* 2006).

When analysing the obtained frequencies of genotypes, in the case of *BTN/HaeIII* polymorphism, considerably higher frequency of *GG* genotype was observed among Jersey cows compared to the Holstein-Friesian cows, in which *GG* genotype occurred very rarely (unpublished results) or was completely absent (HUSAINI *et al.* 1999, KOMISAREK and DORYNEK 2002). The obtained values are similar to the values obtained previously by other authors (KOMISAREK *et al.* 2006b). In the case of the second polymorphic site (*BTN/SchI*), the frequencies of individual genotypes and alleles were similar to those for *BTN/HaeIII* polymorphism, however, incomplete linkage between the studied sites was observed, despite relatively small distance between them. It is also worth mentioning that homozygotic genotype *BTN/SchI*<sup>AA</sup> was observed in twice as many individuals (*n*=35) as the parallel genotype *BTN/HaeIII*<sup>GG</sup> (*n*=17).

The results of conducted analysis also showed the existence of statistically significant differences in the level of production traits depending on genotype. The highest yield of milk was observed in the cows with *BTN/HaeIII*<sup>GG</sup> genotype (*P*≤0.01), but unfortunately, among

animals with that genotype, lower percentage fat content in milk ( $P \leq 0.05$ ) was simultaneously observed in comparison with individuals with *BTN/HaeIII<sup>AA</sup>* genotype. Nevertheless, cows with *BTN/HaeIII<sup>GG</sup>* genotype were characterized by over 11 kg higher fat yield than individuals with *BTN/HaeIII<sup>AA</sup>* genotype ( $P \leq 0.01$ ).

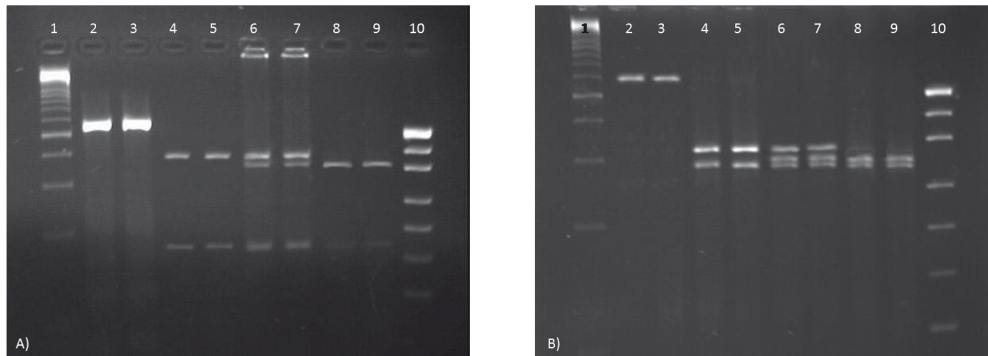


Figure 2

Results of PCR-RFLP analysis

A) *BTN/HaeIII*: 1 = mass marker DNA GenRuler DNA Ladder Mix (Fermentas), 2, 3 = PCR product (568 bp) 4, 5 = AA genotype (371 and 162 bp), 6, 7 = AG genotype (371, 338 and 162 bp), 8, 9 = GG genotype (338 and 162 bp), 10 = mass marker pUC19/MspI 23 (Fermentas). Restriction fragments 10, 12, 13 and 33 bp long were invisible on gel.

B) *BTN/SchI*: 1 = mass marker DNA GenRuler DNA Ladder Mix (Fermentas), 2, 3 = PCR product (568 bp); 4, 5 = AA genotype (315 and 253 bp), 6, 7 = AG genotype (315, 264 and 253 bp); 8, 9 = genotyp GG genotype (264 and 253 bp), 10 = mass marker PUC19/MspI 23 (Fermentas). Fragment 51 bp long was invisible in the photograph.

*Ergebnisse der PCR-RFLP-Analyse*

The analysis of investigated milk performance traits in a configuration of combined genotypes showed the existence of influence of this genotypes on percentage fat contents in milk. The highest fat content (5.66 % and 5.65 %) was observed among individuals possessing the most frequently occurring combination of genotypes of *BTN* gene respectively (*AG/AG*,  $n=51$  and *AA/GG*,  $n=49$ ). The *GG/AG* combination of genotypes deserves special attention, because it seems to negatively influence fat content in milk (5.21 %). However, in the analysed population, only two individuals were carrying this genotypes combination. Simultaneous analysis of the two *BTN* genotypes seems to be appropriate, because those genotypes are not isolated from each other and, in nature, function together within one organism.

Information stored in DNA determines the primary structure of protein. Other levels of protein organization depend largely on the amino acids sequence in the polypeptide chain. Point mutations located in exons may, although not always, result in changes in the primary structure of protein. Butyrophilin protein is composed of three functional domains. On the side of the cytoplasm, just behind the transmembrane region, at the C-terminus of polypeptide chain, a highly conservative domain, known as B30.2, is located. This region represents almost 70 % of cytoplasmatic domain sequence (MATHER 2000).

Nucleotide substitutions investigated in this study (6804 and 6845) are both located in the region of the gene coding B30.2 domain of butyrophilin protein. What is important, the



individual mutations affect the formation of protein with different amino acid composition. In the case of substitution detected using *HaeIII* enzyme, lysine is converted to arginine, whereas the nucleotide substitution in polymorphic site recognized by *SchI* enzyme changes the valine to isoleucine (Table 1). In both cases, there is a conversion to the amino acid with similar properties (BETTS and RUSSEL 2003), so it can be assumed that this substitutions have no significant impact on the structure of proteins of higher order. Moreover, it has been shown that these amino acids are the most frequently mutually substituted in proteins found in cell membranes (BETTS and RUSSEL 2003).

It is not known exactly whether the amino acids are in places directly involved in the ligand binding, hence the need to create a spatial model of protein, useful in clarifying the impact of observed changes in the spatial conformation of endoplasmatic domain. Such a model could also be helpful in recognition and explanation of the molecular mechanism of binding the B30.2 domain with ligand, showing any differences between variants of butyrophilin and the efficiency of their ligand binding, and consequently varied releasing of lipid droplets outside the cell. In the case of mutation, the fat would be released into milk less intensively, which would explain the results received in this work.

The observed lack of linkage between different polymorphic sites is also worth mentioning. The distance between polymorphic sites is only 41 nucleotides, but particular alleles occur with different frequency (Table 1). Because of such small distance, *crossing-over* between analysed places should not occur too often. This leads to the emergence of a different amino acid composition within the B30.2 domain. This seems interesting, given the fact that this domain is considered to be evolutionally conservative, hence the changes within it should not take place. Evolutionary mechanisms only preserve these changes, which give some new beneficial properties to proteins. It is possible that, in the case of butyrophilin, these changes have beneficial impact on the functions of protein.

Complexity of the butyrophilin structure, its not fully understood function, and interesting properties, make the protein a very interesting object of research. In addition, there is still a lot of details to describe concerning its structure and the molecular mechanisms affecting the interaction with other elements responsible for the milk-fat secretion. In conclusion, the obtained results, despite receiving some statistically significant differences, do not appear to be sufficient to render the *BTN* gene variants as useful markers for the selection of dairy cattle, probably because this gene is linked with QTL located in close neighborhood (KOMISAREK and DORYNEK 2002). In addition, because of a low number of individuals in some genotype groups, obtained results need further verification.

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