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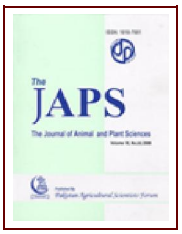

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ABSTRACTING/ INDEXING AGENCIES

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Short Communication
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INFLUENCE OF LEPTIN GENOTYPES ON MILK FAT AND PROTEIN CONTENT OF CROSSBRED HOLSTEIN FRIESIAN X LAI SIND COWS

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ABSTRACT

The aim of this study was to investigate leptin (LEP) gene polymorphisms in crossbred Holstein Friesian (HF) x Lai Sind cattle and their effects on milk yield and milk quality traits. Milk yield was recorded for lactation period of 305 days. Milk fat and protein were estimated using the Milko Tester machine. DNA extracted from blood samples (n=206) was amplified by PCR technique followed by sequencing for single nucleotide polymorphism (SNP) identification. Applied genotyping for the two SNPs (g.C1180T and g.C2059T) was done by PCR-RFLP method with the presence of *Kpn*2I and *Sau*3AI restriction enzymes, respectively. At the LEP/*Kpn*2I locus, cows of CT genotype accounted for the highest proportion (78%) whereas at the second SNP, the frequency of CC dominated (63%). In the association analysis, the LEP/*Kpn*2I polymorphism did not affect 305-day milk yield, but showed significant influence on milk quality traits, of which cows with CT genotype had higher protein (3.67%) and fat (3.99%) content than ones with CC and TT. The other locus (LEP/*Sau*3AI) was not associated with any traits analyzed. In conclusion, the g.C1180T SNP could be included in marker assisted selection of crossbred HF cows for improving protein and fat percentages.

Key words: Cow, leptin gene polymorphism, milk quality trait**.**

INTRODUCTION

Molecular markers contributed greatly to animal breeding along with traditional breeding methods. In dairy cattle, scientists have detected many SNP markers responsible for increased milk yield and milk quality. An example of this is Leptin (LEP), a 4067 bp gene located on chromosome 4 consisting of three exons and two introns (Pfister-Genskow *et al.*, 1996). This gene plays a role in regulating body weight and reproductive function (Frühbeck *et al.*, 2001), growth of breast cells and milk secretion (Hu *et al.*, 2002).

In exon 2 of this gene, Buchanan *et al.* (2002) found a C/T transition leading to the substitution of arginine to cysteine and this tended to change the function of the hormone leptin. By analyzing 416 Holstein cows using *Kpn*2I restriction enzyme, it was concluded that cows with TT genotype produced 1.5 kg/day much more milk than those with CC genotype but little effect on milk fat and protein content was found (Buchanan *et al.*, 2003). On the contrary, Sadeghi *et al.* (2008) found a significant association of this locus with milk yield and milk fat and protein. Another SNP (LEP/*Sau*3AI), previously identified in intron 2 by Pomp *et al.* (1997) was also linked with 305-day milk yield (Moussavi *et al.*, 2006), the T allele provided greater milk yield and reproductive performance. To better evaluate the effect of such alleles in milking cows in Viet Nam, this work was carried out to investigate the distribution of these alleles and their effects on milk traits in crossbred HF x Lai Sind populations.

MATERIALS AND METHODS

Animals: The experiment was conducted on crossbred HF and Lai Sind cattle (F1: 1/2 HF; F2: 3/4 HF; F3: 7/8 HF). All cattle were raised in three different state farms and cooperatives located in the Mekong Delta of Viet Nam.

Data collection: Milk yield of 206 cows was recorded daily throughout the 305-day lactation. Milk samples were collected over three consecutive milking from each cow and milk quality including protein and fat content was measured using the automatic Milko Tester machine (Bulgaria). In addition, blood samples taken from the neck veins were stored in 15 ml falcon tubes containing EDTA anticoagulant and kept at -20^{o}C. Subsequently, the routine phenol/chloroform method was applied to extract DNA (Sambrook and Russell, 2001). The quantity and quality of extracted DNA were tested using spectrophotometric and agarose gel monitoring methods.

Genotyping: The PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism) technique was done for genotyping the animals. At the LEP/*Kpn*2I locus, the forward (5'-ATGCGCTGTGGACCCCTGTATC-3') and reverse primers (5'-TGGTGTCATCCTGGACCTTCC-3') and PCR condition were taken from Buchanan *et al.* (2002).

PCR products of 94 bp were sequenced for SNP detection and incubated with *Kpn*2I restriction enzyme at 37ºC for 15 minutes for genotype determination. In addition, based on the sequence available in GenBank (U50365), another primer pair was designed to amplify a 490 bp fragment containing the second locus LEP/*Sau*3AI. The sequences of forward and reverse primers were: 5'-AAGCTCAGACCTGCAACCAT-3' and 5'-TTGAAAGAGGGCACACACAG-3', respectively. In PCR amplification, an initial denaturation at 94ºC for 3 minutes followed by 35 cycles of denaturation at 94ºC for 45 seconds, annealing at 62ºC for 45 seconds and extension at 72ºC for 45 seconds, and an additional extension of 72ºC for 5 minutes was set. Finally, incubation of PCR products with *Sau*3AI restriction enzyme at 37ºC for 15 minutes was performed to determine the genotypes.

Data analysis: The data were subjected to analysis using General Linear Model of Minitab statistical program version 13.20; factors affecting milk production such as sampling site, animal breed, parity and genotype were included in the model.

RESULTS AND DISCUSSION

Milk yield and milk quality of HF x Lai Sind cows: Milk yield, milk fat and protein content are shown in Table 1. In general, F2 and F3 cows produced similar milk yield (3220 and 3216 kg/305-d lactation, respectively) and had a tendency to produce more milk than the F1 cows (2923 kg/305-d lactation). Theoretically cow breeds with higher HF blood ratio would have higher yield, however as mentioned by Trach and Long (2008), the higher HF blood ratio requires better nutrients and environmental conditions to fully exploit the potential milk production. Thus, it might be one of the factors limiting the milk production potential of F3 cows in the present study.

Table 1. Descriptive statistics of traits analyzed in 305-d lactation (mean ± standard deviation)

Cow group	n	Milk yield (kg)	Fat		Protein	
			%	Yield (kg)	%	Yield (kg)
F1 (1/2 HF)	85	2923±841	4.00±0.51	115.8±33.0	3.66±0.19	107.1±31.8
F2 (3/4 HF)	82	3220±761	3.97±0.51	127.8±34.6	3.60±0.21	115.7±26.4
F3 (7/8 HF)	39	3216±641	3.86±0.55	124.6±33.5	3.55±0.27	114.1±23.6
All groups	206	3097±785	3.96±0.52	122.3±34.0	3.62±0.22	111.9±28.5

In addition, milk fat and protein content tended to decrease according to the higher ratio of HF blood. Fat percentage reached 4% in F1 cows while that of F2 and F3 cows was 3.97% and 3.86%, respectively. This result was in line with the report of Trach and Long (2008), who pointed out that milk fat and protein ratios reduced when HF blood ratio increased, specifically fat ratio decreased from 4.26% in F1 to 3.91% in F2 and 3.77% in F3 cows. Previously, Trach (2003) also noted that HF cows bred in Viet Nam had lower milk fat percentage, and F1 cows usually had higher milk quality as compared with that of F2 and F3 cows.

SNP identification and genotyping: By comparative sequencing, two point mutations of g.C1180T and g.C2059T were identified (Figure 1). These polymorphisms were subjected for genotyping by PCR-RFLP as illustrated in Figure 2. Genotyping by *Kpn*2I restriction enzyme has shown that uncut PCR products with length of 94 bp were TT; those with lengths of 75 and 19 bp were CC and fragments of 94, 75 and 19 bp were of CT genotype (Figure 2a). At the g.C2059T locus, there were 2 cutting sites of *Sau*3AI enzyme in the amplified 490 bp fragment and thus all PCR products were cut into two bands (257 and 233 bp) (Figure 2b). In animals carrying CC genotype, only two bands appeared in the gel while for TT homozygous individuals, the 233 bp band was cut into two fragments of 148 and 85 bp; finally, CT heterozygous cattle showed three fragments of 233, 148 and 85 bp but the 85 bp band was not seen on 2% gel.

At the LEP/*Kpn*2I locus, Buchanan *et al.* (2002) described a mutation point in *Bos taurus* breeding cows namely Angus, Charolais, Hereford and Simmental. Similar findings were proposed in various breeds such as Limousin x Friesian, Simmental x Friesian and Jersey cows (Konfortov *et al.*, 1999) or on Iranian Holstein bulls (Sadeghi *et al.*, 2008). Besides, the LEP/*Sau*3AI polymorphism was also revealed by Javanmard *et al.* (2008) on the Iranian Sarabi cattle, but different primers were used to amplify a 422 bp product. The present data were consistent with those shown by Kulig *et al.* (2009) on Jersey breed and Javanmard *et al.* (2010) on Iranian Holstein breed.

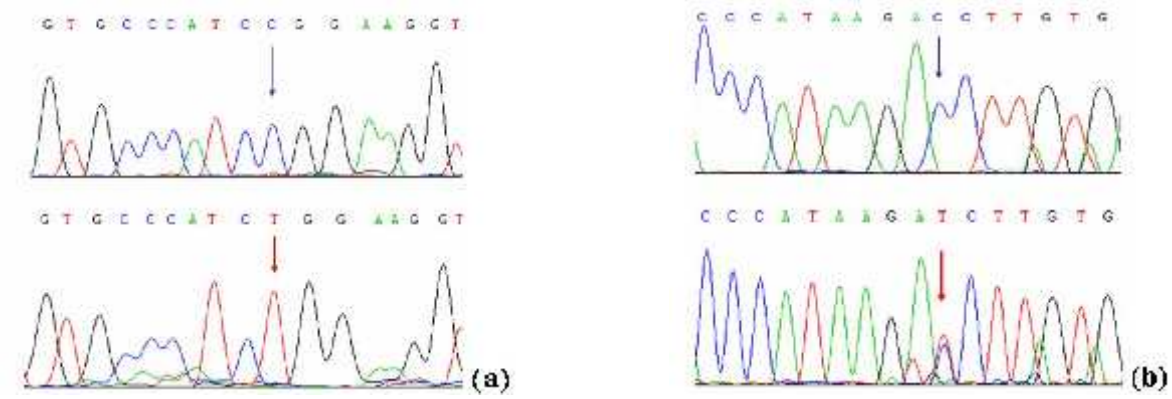

Figure 1. SNP detection by sequencing, arrows show polymorphic sites: (a) g.C1180T and (b) g.C2059T

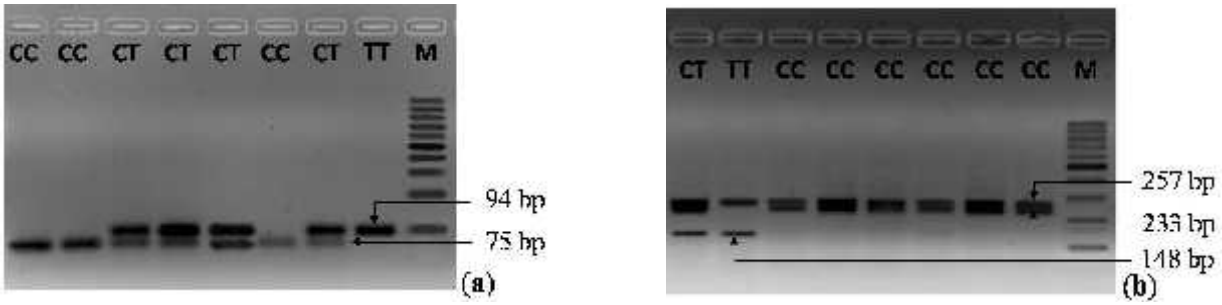

Figure 2. Agarose gel electrophoresis of PCR-RFLP: (a) LEP/*Kpn*2I, g.C1180T and (b) LEP/*Sau*3AI, g.C2059T; M: 100-bp DNA ladder, Fermentas

Table 2. Genotype and allele frequencies of two SNPs in LEP gene

Locus	Genotype frequency (n)			Allele frequency	
	CC	CT	TT	C	T
LEP/*Kpn*2I, g.C1180T	0.12 (24)	0.78 (162)	0.10 (20)	0.51	0.49
LEP/*Sau*3AI, g.C2059T	0.63 (73)	0.23 (27)	0.14 (16)	0.75	0.25

Genotype and allele frequencies of LEP gene polymorphisms are shown in Table 2. At the LEP/*Kpn*2I locus, cows with CT heterozygous genotype accounted for the highest proportion (78%). For the LEP/*Sau*3AI locus, the frequency of CC homozygous genotype dominated (63%), followed by CT and TT genotypes.

In the investigation of Nassiry *et al.* (2007) on the LEP/*Kpn*2I polymorphism, it was shown that CC and CT genotypes appeared on both Golpayegani and Taleshi breeds, while TT genotype was present only on Taleshi breed. Furthermore, Choudhary *et al.* (2005) demonstrated that the percentage TT genotype in HF breed was very low, but relatively high in the Jersey breed. In this study, similar findings were observed, for example in HF crossbred cows, individuals carrying TT genotype appeared in low frequency.

Influence of leptin genotypes on milk yield and milk quality traits: In terms of *Kpn*2I polymorphism, in F1 group, cows with CT genotype had the highest milk yield in 305-day lactation (3114 kg), followed by the TT genotype (2959 kg) and CC (2500 kg) ($P<0.05$). However, in F2 and F3 groups, no differences were found between cows carrying different genotypes (Table 3).

In considering milk quality parameters, at the LEP/*Kpn*2I locus, milk fat and protein percentages were of significant difference ($P<0.05$), in which milk protein was highest with CT genotype (3.67%) and lowest with the CC genotype (3.51%). In addition, CT and TT genotypes had similar milk fat content, which was higher than that in the remaining genotype. As a result, the *Kpn*2I polymorphism significantly associated with milk fat and protein yield in the lactating period ($P<0.05$). In details, fat yield was highest in cows of CT genotype (130.7 kg) and lowest with CC genotype (112.0 kg) and protein yield was highest in cows with CT genotype (119.8 kg), followed by TT (114.8 kg) and CC genotype (105.8 kg).

Table 3. Effects of LEP/*Kpn*2I genotypes on milk production and milk quality traits (least square mean ± standard error)

Cow group	Genotype (n)	Milk yield (kg)	Fat		Protein	
			%	Yield (kg)	%	Yield (kg)
F1	CC (15)	2500±185^{b}	3.82±0.12	96.4±8.8^{B}	3.62±0.05^{b}	90.2±7.0^{B}
(1/2 HF)	CT (58)	3114±110^{a}	4.07±0.07	127.6±5.2^{A}	3.73±0.03^{ab}	116.1±4.2^{A}
	TT (10)	2959±243^{b}	4.08±0.16	120.9±11.7^{AB}	3.80±0.06^{a}	112.4±9.2^{AB}
F2	CC (5)	3342±322	3.54±0.27	119.4±15.6	3.46±0.09^{B}	116.6±12.0
(3/4 HF)	CT (70)	3381±108	3.97±0.09	133.9±5.2	3.70±0.03^{A}	124.9±4.0
	TT (8)	3311±270	4.08±0.22	134.3±13.1	3.49±0.07^{B}	115.6±10.1
F3	CC (4)	3894±307	3.48±0.35	138.9±17.2	3.45±0.11	134.9±11.5
(7/8 HF)[1]	CT (34)	3222±144	3.82±0.16	124.0±8.1	3.61±0.05	116.1±5.4
	TT (2)	2980±403	3.73±0.46	114.1±22.6	3.65±0.14	109.5±15.0
	CC (24)	3019±142	3.70±0.11^{b}	112.0±6.9^{b}	3.51±0.04^{B}	105.0±5.3^{b}
All groups	CT (162)	3272±57	3.99±0.05^{a}	130.7±2.8^{a}	3.67±0.02^{A}	119.8±2.1^{a}
	TT (20)	3181±153	4.03±0.12^{a}	128.0±7.4^{a}	3.61±0.04^{AB}	114.0±5.7^{ab}

[1] *Due to low number of TT animals, statistical analysis is not available*

$^{A-B, a-b}$ *Within each column for each cow group, values with different superscripts are significant different at: small letter (P<0.05), capital letter (P<0.01)*

At the LEP/*Sau*3AI locus, different genotypes did not affect any milk traits examined (P>0.05) (Data not shown).

There have been studies explaining the association between the *Kpn*2I polymorphism and milk traits. For example, Liefers *et al.* (2002) proved that breast epithelial cell lines nurtured in an environment with the presence of leptin had less consolidation of [^{3}H-thymidine], synonymous to DNA synthesis reduction. In other words, leptin negatively affected the development of mammary gland. Thorn *et al.* (2006) indicated that bovine breast epithelial cells had insignificant receptor expression in leptin gene and did not respond to leptin in *in vitro* studies. Leptin therefore cannot affect milking by directly impacting on the epithelial cells. Hence, changes in milk yield recorded in different dairy leptin genotypes might relate to the use of nutrients, in which CT cows were prioritised to produce milk and milk compositions, whereas CC cows favoured accumulation, leading to differences in serum leptin concentrations in later stages of lactation (Liefers *et al.*, 2003).

The current work was consistent with the report of Madeja *et al.* (2004), who found no link between *Kpn*2I and *Sau*3AI polymorphisms and milk production traits. However, this was in contrast with the conclusion of Liefers *et al.* (2002) that *Sau*3AI polymorphism can be used as molecular markers for milk protein yield and milk yield. It was also noted that CT genotype gave higher milk yield of 1.32 kg/day and higher consumption of 0.37 kg/day as compared with CC genotype (Liefers *et al.*, 2003). Buchanan *et al.* (2003) confirmed the remarkable effects of *Kpn*2I polymorphism (TT genotype) on milk and protein yields in the early stages of lactation, homozygous CC cows had lower milk yield than cows carrying CT and TT genotypes. Therefore, the authors proposed that the genotype of LEP, especially T allele, could have played an important role in regulating milk yield and milk protein. These observations were partly validated by this study, where cows with T allele improved milk protein content but had little effect on milk production. All together, it can be concluded that in the HF x Lai Sind population, cows with T allele would be of interest for improving milk protein and fat yield.

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