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GENETIC VARIATION OF THE HEART FATTY ACID-BINDING PROTEIN GENE AND THEIR ASSOCIATIONS WITH MEAT QUALITY IN NATIVE BLACK PIG OF VIET NAM

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Abstract

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The objective of this work was to investigate the genetic variation of heart fatty acid-binding protein (*H-FABP*) gene and their associations with meat quality in Vietnamese Native black pigs. For these purposes, 242 pigs from one Native black pig (NBP) breed (n = 132) and two exotic breeds of Landrace (n = 55) and Yorkshire (n = 55) were included in the analysis of the polymorphisms of a fragment on intron 2 of *H-FABP* gene by the sequencing and PCR-RFLP method. The effects of genotypes on intramuscular fat (IMF) content and backfat thickness (BFT) were evaluated in 105 NBPs. The sequencing results detected in NBP seven single nucleotide polymorphisms, of which the mutation T1556C created a new restriction site for the enzyme *Msp*I. Genotyping *H-FABP* by *Msp*I RFLP method had detected three alleles *A*, *a* and *B* in NBPs, whereas in exotic breeds of Yorkshire and Landrace only two alleles *A* and *a* were found. The frequency of new allele *B* in NBPs was 0.09. The allelic frequencies scored for *H-FABP* gene at *Hae*III site are significantly different between NBP and two exotic breeds ($p<0.01$). Evaluating the effects of genotypes at *Msp*I and *Hae*III loci on IMF content and BFT in NBPs indicated that the novel SNP at position 1556th bp was significantly associated with IMF ($p<0.05$). Allele *B* seemed to be associated with increase in intramuscular fat. However, additional work to confirm the use of this marker in selection programmes is needed.

Key words: *H-FABP* gene, SNP polymorphisms, meat quality, Vietnamese pigs, IMF

Introduction

Pig breeds of Vietnam have been traditionally selected for adaptation to a diet poor in protein content and to the local ecologies. The local pig breeds are said to have some favourable traits like adaptation to tropical climate, disease resistance, good meat quality (Xuan et al., 1995). In recent decades, the pig production in Vietnam was directed to increase the lean percentage of carcass and high growth performance by making use of heterosis mainly between indigenous pig breeds and imported breeds. However, this effort resulted in a decreased meat quality, pork becomes tougher and less flavourable (Molenat and Thong, 1991). Meat quality is an essential trait in meat-producing pigs, therefore qualitative research on meat is becoming more important. Studies have shown that intramuscular fat (IMF) content is one of the most important traits influencing eating quality characteristics (Fernandez et al., 1999). A higher IMF content has a positive effect on the juiciness, palatability and tenderness of meat (Daszkiewicz et al., 2005). Intramuscular fat content, with other meat quality traits, can only be measured on slaughtered animals, which implies costly sib testing to benefit breeding programs. In this respect, marker assisted selection is very promising for breeding programs because information can be determined on living animals at an early age. Among candidate genes investigated for muscle quality, the heart fatty acid-binding protein (*H-FABP*) gene is regarded as the candidate gene for IMF (Gerbens et al., 2000).

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In analysing the effects of the genotypes of *H-FABP* gene on IMF content, it has been found that the genotype of *aa* and *dd* have a higher IMF (Gerbens et al., 2001; Pang et al., 2006). The alleles *a* and *d* are the favourable ones in terms of meat quality. The Native black pig (NBP) is particularly kept by the farmers in the mountainous areas of North Vietnam. This breed is very well adapted to the harsh conditions in the mountainous areas in northern Vietnam and is said to have some favourable traits like good meat quality, adaptation to environmental factors (Trung and Duc, 2008). The NBP breed in the mountainous areas is planned to be mainly preserved as a sow line for crossbreeding with exotic breeds.

With the aims to develop genetic markers to assist the selection for meat quality, the polymorphisms in *H-FABP* gene in Vietnamese NBP breed and two imported exotic breeds of Yorkshire (YV) and Landrace (LV) were investigated by sequencing and genotyping. The association of the *H-FABP* gene polymorphisms with meat quality in NBPs was estimated.

Materials and Methods

Animals and carcass data: For polymorphism, in total 242 unrelated pigs from NBP and two exotic breeds of YV and LV were included in the analysis. The exotic breeds of YV and LV were introduced to Vietnam since 1936 and 1994, respectively (Thien et al., 1996). Blood samples were collected from jugular veins of living animals into test tubes containing an anticoagulant solution. Samples from YV (n = 55) and LV (n = 55) were collected from sows at a breeding station belonging to National Institute of Animal Husbandry (Hanoi). For NBPs (n= 132) samples were collected in Ha Giang, Bac Can and Lao Cai province. For association studies, the samples were collected only from the NBPs. In total 105 offsprings from mating between boars and sows NBPs in Ha Giang (Lung Pu pigs) were used. These pigs were selected based on heterozygosity of boars and sows for the *H-FABP Msp*I and *Hae*III PCR-RFLP to produce litters containing all respective genotype classes. The genotyped offsprings for *H-FABP Msp*I and *Hae*III -RFLP were housed in groups at a commercial station and were given ad libitum access to feed until a slaughter weight of about 75 - 80 kg. Approximately equal numbers of males and females were included. Samples of the dorsal muscle (*m. longissimus dorsi*) were obtained for IMF content evaluation according to Hovenier et al. (1992). Intramuscular fat content was determined using the Soxhlet petroleum-ether extraction method and expressed as the weight percentage of wet muscle tissue. Backfat thickness (BFT) was measured *in vivo* by an ultrasonic instrument (Renco Cooperation, Minneapolis) at defined points on each side of the back, 4.5 cm lateral from the dorsal line (between the third and fourth ribs). Backfat thickness was also recorded after slaughter.

PCR-RFLP genotyping: Genomic DNA from blood and muscle tissue was isolated according to standard protocols (Sambrook et al., 1999). For amplification of target DNA sequence involving a potential polymorphic site, the polymerase chain reaction (PCR) was used. Based on the published nucleotide sequence information of the *H-FABP* gene, the following primers were used to amplify the target DNA sequences of 816 bp: forward primer 5'-ATT GCC TTC GGT GTG TTT GAG-3' and reverse primer 5'-TCA GGA ATG GGA GTT ATT GG-3' (GenBank accession No:Y16180); PCR amplifications were performed in a 25 µl reaction solution containing 2.5 µl 10x PCR Buffer, 2.0 µl 25 mM $MgCl_2$, 0.2 µl 25 mM each dNTPs, 1.0 µl of each 5 pM primer, 0.1 µl 1 u/µl Taq (Fermentas), 1.0 µl 50 ng/µL DNA template and 17.2 µl H_2O deionized. The PCR thermal cycles were 94^0C (4 min), then followed by 35 cycles of denaturation 94^0C (35 s); annealing 57^0C (50 s); polymerization 72^0C (45 s). The final cycle was concluded by an extension interval of 7 min at 72^0C and the PCR products were storaged at 4^0C. Fifteen µl of the PCR products were digested overnight at 37^0C by *Msp*I (6U) and *Hae*III (3U). The digested products were separated by electrophoresis through a 2.5% agarose gel.

Cloning and sequencing: The PCR products were purified with the QIAquick PCR Purification kits (QIAGEN). For sequencing of the RFLP fragment of *H-FABP* gene, the PCR products were cloned into pCR2.1 TOPO vectors (Invitrogen). The positive clones were sequenced by using ABI PRISM® 3100 Avant Genetic Analyzer. The sequences were aligned by Clustalx2, Bioedit version 7.1.3.0, and Mega 4.1 software to identify the mutation sites. Restriction endonuclease DNASTARsites were detected by using primer premier 5.0 software (Lalitha, 2000).

Statistical analysis: Frequencies of genotypes and alleles were estimated by direct counting in three breeds under study. Differences between frequencies of genotypes and alleles were estimated by T- test. Hardy-Weinberg equilibrium (HWE) was tested by comparing expected and observed genotype frequencies using a Chi-square test. If the observed and expected counts were not significantly different ($p>0.05$), the population was in Hardy-Weinberg equilibrium. The effects of polymorphic variants of the *H-FABP* gene on IMF content and BFT traits were tested by the random effects model: $Y_{ij}=m+g_j+e_{ij;}$ where Y is the observed trait, m is the overall mean, g is the group genotype (j=1,k) and e is the residual for the i-th individual (i=1,105). For multiallelic locus, the comparisions were performed by combining the values for animals carrying at least one copy of the relevant allele versus animals with zero allele.

Results and Discussion

Polymorphisms of *H-FABP* gene: By sequencing of a fragment on intron 2 of *H-FABP* gene in NBPs seven SNPs were found; of which five were substitutions: C1438T, T1489C, T1556C, G1811C, C1970T. The frequency of mutation was 0.86% (7/816). Two insertion mutations caused an increasing the size of 816 bp RFLP fragment to 821 bp by adding one and four nucleotides at position 1449 and 1906, respectively. A T/C substitution at position 1556th bp was recognized by the restriction enzyme *Msp*I, so two recognition sites for *Msp*I, at 1489th and 1556th bp, on intron 2 of *H-FABP* gene were created. In addition, a C/G mutation at position 1811th bp, which resulted in removing recognition sites for restriction enzyme *Hae*III, was found through the sequencing the RFLP fragments of *H-FABP* gene (Figure 1). The *Msp*I and *Hae*III RFLP polymorphisms of *HFABP* gene were investigated in NBP, YV and LV. In the NBPs, two *Msp*I recognition sites were found in the RFLP fragments, while in two exotic breeds YV and LV only one *Msp*I site was detected. So in NBPs, digestion of the PCR product with *Msp*I could produce four alleles *a*, *A*, *b* and *B*. In our study, three alleles *a*, *A* and *B* were found by genotyping 132 NBPs (Figure 2). In exotic YV and LV breeds, by genotyping 110 pigs only two alleles *a* and *A* were obtained. Allele and genotype frequencies estimated at *Msp*I loci of the *H-FABP* gene in three pig breeds are given in Table 1. The results showed that the *Msp*I polymorphic site of *H-FABP* gene was found in all three tested breeds. The allele *B* appeared only in NBPs with the frequency of 0.09. Both in indigenous and exotic breeds, allele *A* was a major allele. The frequencies of favorable allele *a* were not much different between indigenous and exotic breeds. The genotype frequencies at *Msp*I loci obtained in three tested breeds were in Hardy-Weinberg Equilibrium.

Heart fatty acid-binding protein plays an important role in the transportation of intracellular fatty acids thereby affects the intramuscular fat content (Cameron et al., 1991). Three restrictions enzyme cut loci (*Hinf*I, *Hae*III, *Msp*I) of

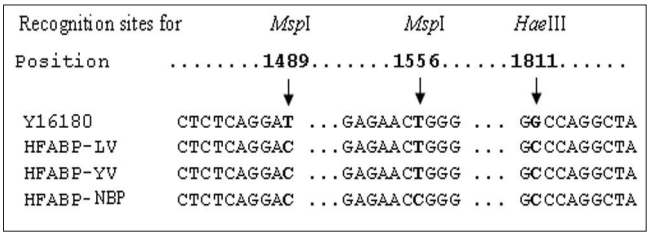

Fig. 1. Recognition sites for *Msp*I and *Hae*III on intron 2 of *H-FABP* gene

Blast results of the nucleotide sequence in three pig breeds with nucleotide sequence in GenBank accession No:Y16180. NBP: Native black pig; LV: Vietnamese Landrace; YV: Vietnamese Yorkshire

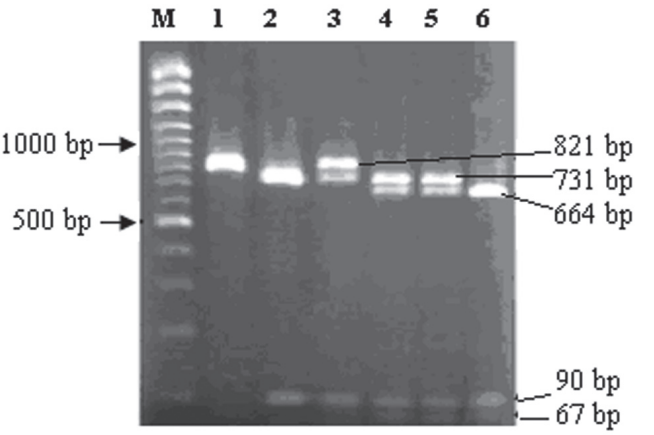

Fig. 2. *Msp*I polymorphism of the *H-FABP* gene.

The genetic variations of *H-FABP* gene were investigated by PCR-RFLP. The digested PCR products with *Msp*I generated a 821 bp fragment for allele *a*; 731, 90 bp for allele *A*; 664, 157 bp for allele *b*; and 664, 90, 67 bp for allele *B*. Lane M: standards for size determination. Lane 1: PCR product of 821 bp. Lane 2: genotype *AA*. Lane 3: genotype *Aa*. Lane 4, 5: genotype *AB*. Lane 6: genotype *BB*

Table 1
Genotype, allele frequencies of *H-FABP* gene in Native black pigs, Landrace and Yorkshire breeds at *MspI-RFLP* site

Breed[a]	Sample size (n)	Genotype frequencies (No. of genotypes)[b]				Allele frequencies			$\chi 2^c$
		AA	*Aa*	*AB*	BB	A	a	B	
NBP	132	0.80 (106)	0.04 (5)	0.14 (19)	0.02 (2)	0.89	0.02	0.09	NS
LV	55	0.91 (50)	0.09 (5)	-	-	0.95	0.05	-	NS
YV	55	0.95 (52)	0.05 (3)	-	-	0.97	0.03	-	NS

[a]Abbreviations of pig breeds: NBP: Native black pig; LV: Vietnamese Landrace; YV: Vietnamese Yorkshire. [b] Values in parentheses are the numbers of pigs found in each genotype. [c] Chi-square test. NS: Not significant (the population was in Hardy-Weinberg Equilibrium)

H-FABP gene were identified as RFLP markers for IMF in pigs. For this reason the RFLP fragments on intron 2 of *H-FABP* in local NBP breed, which is said to have good meat quality, were sequenced. The results revealed seven mutations in comparison with the reference sequence (GenBank accession No:Y16180). The average distance among these single nucleotide polymorphisms (SNPs) was about 102 bp. Corresponding values of 81.7 bp have been observed by screening SNPs in *H-FABP* gene promoter region in seven Chinese indigenous pig breeds (Zhang et al., 2011). In NBP breed, the frequency of new allele *B* at position 1556th was 0.09, higher than the favourable allele *a* (0.02) at position 1489th bp ($p<0.05$). These findings correspond with the previous results, that allele *B* was found only in Vietnamese pig breeds of Meo, Muong Khuong, Tap Na with the frequencies of 0.01, 0.11 and 0.07, respectively (Cuong et al., 2012). The **a**ppearance of new allele *B* in some Vietnamese pig breeds may be caused by selection on distinct characteristics e.g. a diet poor in protein content. Such selection has driven the accumulation of new mutations with favorable phenotypic effects, as well as the development of alleles and haplotypes that differ from exotic breeds (Thuy et al., 2006). Analysing genetic variants at the *H-FABP* locus in the Australian pigs Nechtelberger et al. (2001) revealed also a new restriction site for *Msp*I at this locus. A new SNP (T/C) at -224 bp, named *H-FABP*-proT224C, was found in *H-FABP* gene promoter region (Erdun et al., 2008). In this study, allele *b* was not detected by genotyping 132 NBPs. A higher frequency of allele *A* at the *Msp*I RFLP site was observed in indigenous and exotic breeds. There were no significant differences between indigenous and exotic breeds with respect to the distribution of allele frequency at 1489th bp locus *Msp*I.

The same 816 bp fragment had three *Hae*III sites that could produce four fragments (405, 278, 117 and 16 bp). A polymorphism at the position 1811th bp would result in the destruction of a *Hae*III site, merging the 278 bp and 405 bp into a 683 bp fragment. Allele *d* was defined as the presence of 405, 278, 117 and 16 bp fragments. Allele *D* is the digested PCR products at two positions to create three fragments of 683, 117 and 16 bp. Allele and genotype frequencies estimated at *Hae*III loci are given in Table 2. In NBP breed, allele *D* was a major allele, with frequency of 0.92, meanwhile in exotic YV and LV breeds, the allele *D* was detected with a frequency of 0.42 and 0.65, respectively. The genotype distributions at *Hae*III locus obtained in three tested breeds were in Hardy-Weinberg Equilibrium.

At locus *Hae*III, the distribution of allele frequency was significant different between NBP and two exotic YV and LV breeds ($p<0.01$). The favorable allele *d*, which was reported to have a positive effect on IMF content, in exotic breeds, was higher than those of indigenous breed ($p <0.01$). These findings correspond with the results of Pang *et al*. (2006); these authors revealed that in Landrace and Chinese native pig breeds the frequencies of allele *A* at *Msp*I -RFLP site were high, with the value of 0.912 and 1.0, respectively; meanwhile the frequencies of allele *D* at *Hae*III site were 0.328 and 1.0, respectively.

Association of *H-FABP* genotypes with IMF and BFT: The effect of *H-FABP* genotypes was estimated both for IMF content and BFT in NBPs. As presented in Table 3, pigs with allele *B* exhibited significantly heigher IMF content when compared with alleles *A* and *a* ($p<0.05$). Apart from allele *B*, no significant differences in IMF, however, was revealed between any of the alleles at *Msp*I loci in this study. The statistical results suggest that the SNP of T1489C did not affect IMF content, because animals with the genotype *Aa* showed no significant difference on IMF content to genotype *AA*. For the *Hae*III RFLP, there was no difference in IMF content between three genotypes *DD*, *Dd* and *dd* (Table 4). The value of BFT in NBPs was high and varied from 33.57 mm (*AB*) to 34.85 mm (*AA*). Both *Msp*I and *Hae*III RFLP, there was no significant effect of the *H-FABP* genotypes on backfat thickness.

Recent cytogenetic mapping of the *FAB* gene family showed that a cluster of three genes, including *H-FABP* gene,

Table 2
Genotype, allele frequencies of *H-FABP* gene in Native black pigs, Landrace and Yorkshire breeds at *Hae*III-RFLP site

Breed[a]	Sample size (n)	Genotype frequencies (No. of genotypes)[b]			Allele frequencies		$\chi 2^c$
		DD	*Dd*	*dd*	*D*	*d*	
NBP	132	0.88 (116)	0.08 (11)	0.04 (5)	0.92	0.08	NS
LV	55	0.22 (12)	0.40 (22)	0.38 (21)	0.42	0.58	NS
YV	55	0.435 (24)	0.435 (24)	0.13 (7)	0.65	0.35	NS

[a]Abbreviations of pig breeds: NBP: Native black pig; LV: Vietnamese Landrace; YV: Vietnamese Yorkshire. [b] Values in parentheses are the numbers of pigs found in each genotype. [c] Chi-square test. NS: Not significant (the population was in Hardy-Weinberg Equilibrium)

are tightly linked with IMF (Szczerbal et al., 2007). Gerbens et al. (1999, 2000) have elucidated that SNPs at the position 1489th bp and 1811th bp were associated with intramuscular fat content. This effect of 0.36% IMF between either homozygous genotype classes is high considering the overall mean of 1.84% IMF in the Meishan crossbred population. The single marker association analysis in Large White pigs showed that the *H-FABP* gene was associated with IMF (Han et al., 2012). In the Chinese local pig breeds, the IMF content ordered by *H-FABP* genotypes were *HH>Hh>hh, DD<Dd<dd*, and *AA<Aa<aa* (Wei-Jun et al., 2006). In contrast, no association was detectable in Australian Large White, Piétrain, and Landrace (Nechtelberger et al., 2001). In Polish native Złotnicka Spotted pigs, the meat with genotype *hh* were marked by a higher intramuscular fat content (2.64%) compared to *HH* group (Hanna et al., 2010). The IMF deposition is a complicated process, affecting by genetic, physiological, and biochemical factors. Intramuscular fat content has a higher heritability (0.6) (Hermesch et al., 2000). With respect to genetic factors, 4 to 13 QTLs for IMF content have been identified in different pig populations (Gao and Zhao, 2009). Switonski et al. (2010) reported that there are 29 genes, which are associated with IMF content. Intramuscular fat content depends on the genotypes and on the breeds (Fisher, 1994). Pang et al. (2006) found that the values of IMF content in European and Chinese breeds are significantly difference, although they have the same genotype. Significant associations between the single SNP and phenotypic traits were observed in breeds, which showed a higher content of IMF as Duroc. The IMF content in NBPs is high, varied from 3.56 % (*AA*) to 3.97% (*BB*), this may be the reason why in NBPs the polymorphism of *H-FABP* gene is associated with IMF. Interestingly, in NBP breed significant effects of the *H-FABP – Msp*I genotypes on IMF were not observed at position 1489th bp, but at position 1556th bp. There are two possibilities to improve IMF content, by enhancing favorable alleles and by crossbreeding with breeds having higher value of IMF content. The NBP breed has good meat quality due to high rating for overall palatability and tenderness. Marker *Msp*I-RFLP at T1556C position can be used to improve IMF content by enhancing favorable allele *B* and tracking the genetic materials in hybrids. In this study, significant association between the single SNP and IMF content was presented by one factor analysis, but no with the mixed model. Therefore, it was uncertain whether there was any association between this single SNP and IMF content. Haplotype or haplotype block provided a solution to resolve these problems. In addition, the effects of allele *b* on IMF content should be taken into consideration. So further studies are required to confirm the associations of this SNP with IMF content in NBPs.

Statistical analysis show that the variation in the *H-FABP* gene is not associated with BFT, so the effect of *Msp*I *H-FABP* alleles on IMF content is not related to BFT. MspI for IMF and BFT are with a tendency for a negative link, even the BFT differences between genotypes were not significant (Table 3). In addition, Hermesch et al. (2000) found that IMF content has a moderate negative correlation with back fat thickness. These results differed from those published by Gerbens et al. (1999); these authors observed significant effects of the *H-FABP* – MspI genotypes not only on IMF, but also on the backfat. Wei-Jun et al. (2006) found also that fat deposition in adipocytes was stronger in the *dd* and *aa* genotypes than in others.

Table 3
Associations between genotypes of *H-FABP* gene and meat quality at *Msp*I locus in Native black pig breed

Traits	*H-FABP- Msp*I genotypes (No. of genotypes)					
	Aa *n =15*	*AA+Aa+AB* n =95	*AB+BB* *n =50*	*AA+AB+BB* *n =90*	*BB* *n =10*	*AA + Aa* *n =55*
IMF, %	3.59±0.47[a]	3.66±0.43 [a]	3.82±0.46[b]	3.70±0.45 [a]	*3.97*±0.60 [b]	3.57±0.41 [a]
BFT, mm	33.89±3.19 [c]	34.16±3.40 [c]	33.58±3.43[c]	34.15±3.52[c]	33.89±3.28[c]	34.59±3.43 [c]

IMF = Intramuscular fat content; BFT = Backfat thickness. Within rows, the means with different superscripts significantly differ ($p<0.05$)

Table 4
Associations between genotypes of *H-FABP* gene and meat quality at *Hae*III locus in Native black pig breed

Traits	Sample size (n)	*H-FABP- Hae*III genotypes (No. of genotypes)		
		DD (92)	*Dd (8)*	*dd (5)*
Intramuscular fat content, %	105	3.70±0.54[a]	3.86±0.33 [a]	3.91±0.90 [a]
Backfat thickness, mm	105	34.05±3.54[b]	34.41±2.57 [b]	34.69±3.07[b]

Within rows, the means with different superscripts significantly differ ($p<0.05$

Conslusion

A new mutation at position 1556th bp on intron 2 of *H-FABP* gene, which created the second *Msp*I recognition site in RFLP fragment, was found in Vietnamese Native black pigs. At the *Msp*I RFLP sites, three alleles were found in Native black pigs, whereas in exotic Yorkshire and Landrace only two alleles were detected. The novel SNP showed significant association with IMF content. This new mutation may be used as *Msp*I-RFLP marker to improve IMF content by crossbreeding. However, additional work to confirm the use of this marker in selection programmes is needed.

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