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Polymorphisms and Sequencing Analysis in 5'Upstream and Intron 2 Region of *H-FABP* Gene in Anhui Wannan Black Pig Population

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Abstract: In order to give a basis for molecular breeding of meet quality traits in Wannan black pig, we applied PCR-RFLP method to analyze the polymorphism of 5'upstream region and intron 2 of *H-FABP* gene in 184 Wannan black pigs population. The results show that there exist polymorphic loci of restriction enzyme Hinf in 5'upstream region and Hae III as well as Hinf I in intron 2. Except for locus of restriction enzyme HinfI, the other two polymorphism sites achieved in Hardy-Weinberg equilibrium based on χ^2-test ($p>0.05$). In addition, a T→C mutation in 1324 bp, a C→G mutation in 1811 bp and a C→T mutation in 1970 bp were found through the direct sequencing the polymorphic RFLP fragments of *H-FABP* gene, then genotype frequencies, gene frequencies, H_o, H_e, N_e and PIC were investigated in Wannan black pig population. The results identified 3 variant restriction sites and provided a basic data for further study of the relationship between IMF and H-FABP with different genotypes and also provide basic for make sure whether *H-FABP* gene is the key gene for effect on the deposition of IMF.

Key words: Wannan black pig, H-FABP, IMF content, PCR-RFLP, sequencing, China

INTRODUCTION

The most purpose of common breeding program aim to improve lean and growth rate with the decrease of fat, the Intramuscular Fat (IMF) is also reduced and resulting in a decline of quality in pork. IMF which belongs to quantitative traits and be controlled by multiple genes has an influence on flavor, juiciness especially for tenderness of meat (Wood *et al*., 2004). Recently, the selection for lean rate makes the content of IMF to reduce to 1-1.5% while the ideal value is 2-3% (Bejerholm and Barton-Gade, 1986). Reportedly, IMF has a higher heritability (0.6) and has a moderate negative correlation (0.3) with Backfat thickness (Hermesch *et al*., 2000; Hovenier *et al*., 1992). For this reason, we can improve pork quality by carrying on genetic improvement in IMF.

Heart Fatty Acid-Binding Protein (H-FABP) is a member of FA bp family and plays an important role in the transportation of Intracellular fatty acids (Cameron and Enser, 1991). It combined with fatty acid inside the cells, maintaining a certain fatty acid concentration difference inside and outside the cells to improve the intake of fatty acids thereby affecting the deposition of fat (Veerkamp *et al*., 1991). The structural feature of H-FABP has been confirmed and was also mapped at the position of markers SW316-S0003 in chromosome 6 (Gerbens *et al*., 1997, 1999). Genomic sequence is consists of 1.6 kb upstream regulatory regions, 0.2 kb 3' end non-transcribed region, 3 introns with a size of 4.2 kb, 2.5 kb, 1.5 kb, respectively and 4 exons which coding amino acids with a number of 24, 58, 34, 17 and *H-FABP* gene mainly expressed in myocardial, skeletal and breast (Chmurzynska, 2006). Three restrictions enzyme cut loci (HinfI HaeIII MspI) of *H-FABP* gene were found in Duroc population and to be regarded as the candidate gene for IMF. Gerbens *et al*. (2000, 2001) detected the polymorphism of H-FABP mRNA, protein expression level and IMF on the longissimus muscle content based on the tested groups including 153 hybrids of Meishan pig. The results showed that IMF levels of genetic variation had nothing to do with the level of protein expression but had a significant association with mRNA level ($p<0.05$).

In order to provide a foundation for the confirmed of the major gene of IMF deposition for further studies and also provide foundation for the research on the characteristics of breed resources of Wannan black pig population. In this study, we also chosen the Wannan black pigs of Anhui province in China which has a higher level of IMF as the test population and applied the PCR-RFLP method to detect the polymorphisms of 5'upstream region and intron 2 of *H-FABP* gene and then we sequenced the RFLP enzyme cut fragments and

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genotype frequencies, gene frequencies, homozygosity, heterozygosity, effective number of alleles and polymorphism information content of polymorphisms in H-FABP were also investigated.

MATERIALS AND METHODS

DNA samples: Ear tissue samples of 184 Wannan black sows were collected from Anhui province Rich Ecological Agriculture Development Co., Ltd. All samples were stored in 1.5 mL Ep tube filled with 1 mL 70% ethanol at -20°C. Genomic DNA was extracted by applying conventional method (Sambrook *et al*., 1999).

PCR amplification: Through using Primer Premier 5.0 software, two pairs of primer for 5'upstream region and intron 2 were designed according to the genomic sequence of *H-FABP* gene in NCBI (GenBank Accession No.X98558; No.Y16180) (Table 1).

The PCR amplification was carried out in a total volume of 25 μL, including 12.5 μL premix tag (Version 2.0), 2 μL template DNA, 1 μL of each primer and 8.5 μL sterilization distilled water. PCR was performed under the following reaction procedure: initial denaturation at 95°C for 5 min, then followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec and extension at 72°C for 10 min, ending of an extension step at 72°C for 5 min and stored at 4°C.

PCR-RFLP detection and sequencing: The RFLP detection of the amplification products was proceed in a system with a volume of 20 μL, containing 6 μL PCR amplification products, 2 μL 10×Buffer and 1 μL restriction enzyme (HinfI HaeIII MspI) at 37°C for 4 h. After then put these products were detected in 2.5% agarose gel. The PCR products which represented different PCR-RFLP genotype including both homozygous and heterozygous genotypes were purified with the Gel Midi PCR DNA Purification kit (Tiangen Biotechnology, China) and sequenced by Shanghai Biological Engineering Technology Services Ltd. using the ABI377 sequencer.

Table 1: Primer pairs for PCR amplification of *H-FABP* gene

Fragments	Primers	Products size (bp)	Positions
PCR1[A]	5'-GGACCCAAGATGCCTACGCCG-3'	693	1125-1818
	5'-CTGCATCTTTGACCAAGAGG-3'		
PCR2[B]	5'-ATTGCTTCGGTGTGTTTGAG-3'	816	1401-2217
	5'-TCAGGAATGGGAGTTATTGG-3'		

A: The region sequence ranges from 5'-upstream, exon 1 and part of intron 1 (Genebank Accession No.X98558), the enzyme reaction region of Hinf; B: The region sequence is in intron 2 (Genbank Accession No.Y16180), the enzyme reaction region of HaeIII, MspI, HinfI*, HinfI* represent enzyme reaction in intron 2 region

Those tested sequences were aligned by using the DNAMAN software (version 5.2.2) to identify the mutation site.

Statistic analysis: The gene and genotype frequency of the SNP were calculated:

$$P_i = (2(ii)+(ij_1)+(ij_2)+\ldots+(ij_{n-1})+(ij_n))/2$$

Where:

P_i = The frequency of allele i
i_i = The number of homozygous alleles i
j_n = The allele n having codominance with allele i
ij_n = The number of j_n
n = The number of individual of one population

It has been observed that the alleles are codominant due to the results of PCR-RFLP, so that we speculated the phenotype frequency is equal to genotype frequency. After the calculation for frequency, χ^2 test was applied to detect the significant as follows:

$$x^2 = \sum_i^n \frac{(|O_i - E_i| - 0.5)^2}{E_i}$$

Where:

E_i = The theoretical value
O_i = The actual observation value
n = The number of alleles

The population genetic parameters including Homozygosity (H_0), Heterozygosity (H_e), effective number of alleles (N_e) and Polymorphism Information Content (PIC) of *H-FABP* gene were investigated orderly. The formulas as following:

$$H_0 = \sum_{i=1}^{n} P_i^2 \quad (1)$$

Where:

H_0 = The homozygosity of one locus
P_i = The frequency of an allele i
n = The number of alleles

$$H_e = 1 - H_0 = 1 - \sum_{i=1}^{n} P_i^2 \quad (2)$$

where, H_e is the heterozygosity of one locus.

$$N_e = 1/H_0 \quad (3)$$

where, N_e is the effective number of alleles.

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2 \quad (4)$$

Where PIC is a target for measuring the extent of population polymorphism proposed by Botstein *et al.* (1980) showing highly or lowly polymorphic with a threshold of PIC>0.5 or PIC<0.25, respectively.

RESULTS AND DISCUSSION

Polymorphisms analysis: The fragments for 5'upstream and intron 2 region of *H-FABP* gene were successfully amplified (Fig. 1). By applying the PCR-RFLP method, two fragments (693 bp, 816 bp) were detected. There were 4 HinfI enzyme cut loci in fragment 1 (693 bp), resulting in 5 fragments with a length of 339, 172, 98, 59 and 25 bp, respectively. The polymorphism enzyme cut site located in 1324 bp place and a new fragment (231 bp) was came out of a combination of two fragments (172 bp, 59 bp) as the site disappeared (Fig. 2). For the fragment 2 (816 bp), there exist 3 HaeIII, 1 MspI and 3 HinfI enzyme cut loci, Fig. 3 and 4 have given the visualized information as shown in Fig. 3, the polymorphism enzyme cut site is in 1811 bp place and when this site disappeared, the combination of fragments of 278 and 405 bp generated a new fragment of 683 bp.

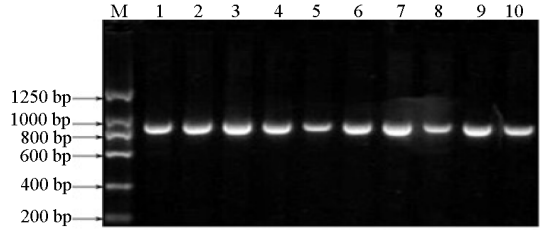

Fig. 1: PCR amplification of *H-FABP* gene; M: DL200 molecular weight marker

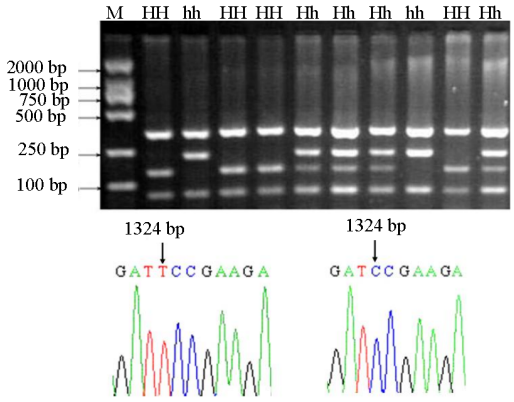

Fig. 2: The electrophoresis patterns of PCR- HinfI-RFLP and sequencing maps for SNP in 5'upstream region of porcine *H-FABP* gene, the SNP are indicated by black arrows; M: DL2000 molecular weight marker

Sequencing of RFLP fragments of *H-FABP* gene: Compared with the results after sequencing for *H-FABP* gene, in the 5'upstream region, the amplification of the 693 bp fragment occur a mutation of T-C in 1324 bp and forming the two alleles H and h (Fig. 2). In intron 2, the amplification of the 816 bp fragment was found a mutation of G-C in 1811 bp and forming the two alleles D and d (Fig. 3). Similarly, a mutation of C-T in 1970 bp also found and resulted in two alleles B and b (Fig. 4).

Population genetic analysis: For the three polymorphism enzyme cut loci, we detected their genotypes and calculated the corresponding genotype frequencies, gene

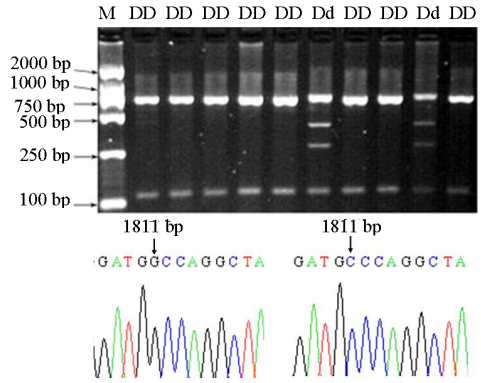

Fig. 3: The electrophoresis patterns of PCR-HaeIII-RFLP and sequencing maps for SNP in intron 2 of porcine *H-FABP* gene, the SNP are indicated by black arrows; M: DL2000 molecular weight marker

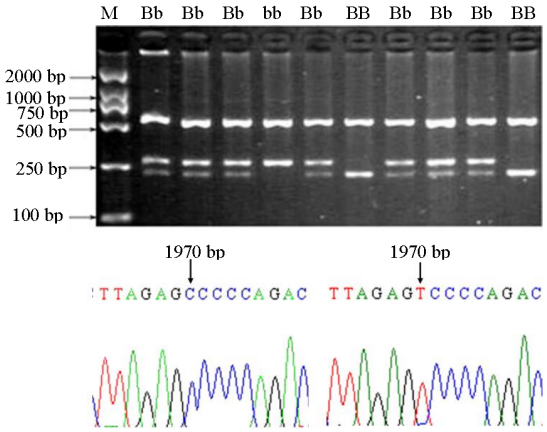

Fig. 4: The electrophoresis patterns of PCR- HinfI-RFLP and sequencing maps for SNP in intron 2 of porcine *H-FABP* gene, the SNP are indicated by black arrows; M: DL2000 molecular weight marker

Table 2: The genotype and gene frequencies of SNPs of the H-FA bp gene in Wannan black pig population

	Genotype artribution								
	HinfI -RFLP			HaeIII-RFLP			HinfI*-RFLP		
Loci genotypes	HH	Hh	hh	DD	Dd	dd	BB	Bb	bb
Numbers of sample	81.00	67.00	36.00	156.00	28.00	0	108.00	72.00	4.00
Genotype frequency	0.44	0.36	0.20	0.85	0.15	0	0.59	0.39	0.02
Dominant gene frequency	-	0.62	-	-	0.92	-	-	0.78	-

frequencies, H_o, H_e, N_e and PIC (Table 2-4). In Table 2, genotypes of HH, DD, AA and BB express a great advantage as well as the frequency of allele H, D, A and B relative to allele h, d, a and b. For Haeβ, only two genotypes (DD, Dd) were detected which indicated a high homozygosity in the two sites. As for Hinf, the polymorphism sites were detected in both 5'upstream region and intron 2. The frequencies of genotypes and genes for 3 polymorphisms were shown in Table 3, except for Hinf polymorphism site, the other two polymorphisms achieve Hardy-Weinberg equilibrium ($p>0.05$), these results indicate a genetic dominance in adaptability and an equilibrium state after a long evolution and selection. The reason for disequilibrium of Hinf may be artificial selection in breeding.

About 2 fragments (693, 816 bp) both present polymorphism after being digested with HinfI and their H_o, H_e, N_e and PIC values all indicate a moderate polymorphism (Table 4). The results also show a low polymorphism for HaeIII site. Reasons for these may caused by the closed environment for these test pigs, result in less blood of other breeds to enter and through long-term nature inbreeding within populations making the continued purification for favorable genes as well as some alleles lost, resulting in the reduction of its PIC. The other reason may also be less samples in the test population.

Based on the foregone research, we can know that every PCR-RFLP fragments detected in 3 enzyme cut polymorphic loci (HinfI, HaeIII, MspI), the contents of IMF have a significant difference between homozygous genotypes and genotypes of aa, dd and HH (MspI-a, HaeIII-d, HinfI-H) have a higher IMF (Gerbens *et al*., 1999, 2000; Pang *et al*., 2006). The content of IMF of pigs in China belongs to a medium level (3-7%). The results showed polymorphism for HinfI in 5'upstream region which may have an influence on the expression of *H-FABP* gene and thus affect the content of IMF (Gerbens *et al*., 1999, 2000). In addition, the higher frequencies of allele A, D, H and B may also result in a higher content of IMF in Anhui Wannan black pig population.

Besides *H-FABP* gene, there also have other genes from QTL region of fat traits such as HSL (GU *et al*., 1992), PGD (Lalley *et al*., 1978) and LEPR genes in chromosome 6. HSL and PGD genes are significant association with backfat thickness and loin eye area traits in pigs (Lei *et al*., 2005; Clamp *et al*., 1992) and the *LEPR* gene may be a potential candidate gene to influence fat traits (Switonski *et al*., 2010). However, further studies were also needed to explore the relationships between these genes combined with more other candidate genes and DNA markers. In order to apply Marker Assisted Selection (MAS) in animal breeding, genetic markers tightly linked with IMF traits should be found to further determine the relationship between IMF and H-FABP with different genotypes. Moreover, the H-FABP is the cultured breast epithelial cell growth inhibitor for cattle, mouse and human (Lehmann *et al*., 1989) so whether the variation of H-FABP is a reason to cause the small size and slow growing in Anhui Wannan black pig population is in progress in our group.

Table 3: Hardy-Weinberg equilibrium analysis for PCR-RFLP site

Site	Chi-square value (χ^2)	p-vaule
HinfI	9.35	0.0093
HaeIII	1.25	0.5358
MspI	-	-
HinfI*	4.14	0.1262

Table 4: Population genetic parameter (H_o, H_e, N_e and PIC) of SNPs of *H-FABP* in Wannan black pig

SNPs	H_o	H_e	N_e	PIC
HinfI -RFLP	0.530	0.470	1.887	0.356
Hae III-RFLP	0.860	0.141	1.164	0.131
HinfI*-RFLP	0.660	0.340	1.516	0.282

CONCLUSION

In this study, polymorphisms of 5'upstream and intron 2 region of *H-FABP* gene were identified in Wannan black pig population, their mutational sequences of 3 restriction sites were also identified. Population genetics parameters including genotype frequencies, gene frequencies, homozygosity, heterozygosity, effective number of alleles and polymorphism information content of SNPs in H-FABP were also investigated. These results may provide basic data for the further molecular breeding of meet quality traits in Anhui Wannan black pig population and give a theoretical basis for looking for more significant major genes which have an effect on the deposition of IMF.

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