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Distribution and linkage disequilibrium analysis of polymorphisms of *MC4R*, *LEP*, *H-FABP* genes in the different populations of pigs, associated with economic traits in DIV_2 line

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Abstract PCR-RFLP was used to analyze the polymorphisms of *MC4R*, *LEP*, *H-FABP* genes in a swine breed composite (DIV_2) and 4 swine breeds (Yorkshire, Landrace, Meishan, Bamei). The association study of these polymorphisms with several economic traits was carried out on a DIV_2 population. The results obtained showed that *MC4R*/*Taq*I genotype had an effect for average backfat thickness ($P < 0.05$) and lean meat percentage ($P < 0.05$). At locus *LEP*/*Hin*fI animals of AA genotype had lower test daily gain than that of BB ($P < 0.01$) or AB genotype ($P < 0.05$). At the *H-FABP*/*Hae*III locus lean meat percentage of the individuals with genotype DD were higher than that with genotype dd ($P < 0.05$). Linkage disequilibrium analysis among *MC4R*, *LEP* and *H-FABP* revealed that these genes were independent. This represented two or more genes that could be combined together within one genotype in order to facilitate breeding for objective traits. In addition, a method allowing simultaneous detection of fragments of *MC4R* and *LEP* gene was developed.

Keywords *MC4R* · *LEP* · *H-FABP* · Multiplex PCR · Linkage disequilibrium

Introduction

Candidate gene markers focus on polymorphisms in a gene that are postulated to affect the trait; they are often tightly linked to the quantitative trait loci (QTL) [1]. These polymorphisms are revealed by molecular techniques such as restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP), single nucleotide polymorphisms (SNPs) and other [2]. Polygenic characters, which were previously very difficult to analyze using traditional animal breeding methods, would now be easily tagged using molecular markers.

Melanocortin-4 receptor (MC4R) is a centrally expressed G-protein coupled receptor encoded by a single exon on porcine chromosome 1. MC4R plays an important role in the control of feed intake and energy homeostasis. A missense mutation, an G → A substitution (within a *Taq*I restriction site) in position 1426 of MC4R, results in the replacement of aspartic acid with asparagine at position 298 of the protein, and has been clearly associated with back fat thickness, feed intake and growth rate [3, 4].

The protein hormone leptin (LEP) is secreted mainly by adipose tissue and encoded by the *LEP* gene [5]. Leptin impacts feed intake, body weight and metabolism through physiological and endocrine mechanisms [6]. In swine, the *LEP* gene was mapped on chromosome 18q13-q21 [7]. A single nucleotide polymorphism (T3469C) was detected in a coding region, in the second exon of *LEP*. This mutation changes the recognition site for enzyme *Hin*fI. Association analysis revealed that the polymorphism was significantly associated with production traits [8].

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Heart fatty acid binding protein (H-FABP) is a member of the family of fatty acid binding proteins. This protein is widely distributed but mainly expressed in heart and skeletal muscle. The critical roles of the FABP family in the transport and metabolism of intracellular fatty acid have been reported [9]. The porcine *H-FABP* gene was localized on chromosome 6. A SNP (within a *Hae*III restriction site) in the second intron was identified in porcine *H-FABP* gene and related to intramuscular fat (IMF) content [10, 11].

The aim of this paper was to provide basic data for marker-assisted selection and theory base for the improvement of genetic characters of the pig. We estimated the frequencies of *MC4R*/*Taq*I, *LEP*/*Hin*fI and *H-FABP*/*Hae*III gene mutations in five pig populations and found possible associations between different genotypes of these genes and economic traits in the Chinese lean-type new line (DIV_2 line). The implications of the allelic distribution of these three genes (*MC4R*, *LEP* & *H-FABP*) for meat production, and applicability of molecular markers in pig breeding were discussed.

Materials and methods

Animals

In this study, 209 DIV_2 pigs, 77 Yorkshire pigs, 65 Landrace pigs, 70 Meishan pigs and 40 Bamei pigs were from the Experimental Pig Station of Huazhong Agricultural University (Wuhan, China). DIV_2 belonged to a synthetic line of Yorkshire, Landrace, Meishan and/or Tongcheng origin.

DNA extraction

Genomic DNA was extracted from blood according to the standard phenol–chloroform method and stored at −20°C [12].

PCR-RFLP

The *MC4R*, *LEP* and *H-FABP* fragments were amplified from a genomic template using PCR with primer sequences (the same primers used for Multiplex PCR-RFLP) reported by Kim et al. [4], de Oliveira Peixoto et al. [8] and Gerbens et al. [10], respectively. PCR amplifications were carried out in a 25 μl reaction mixture containing 1 μl (50 ng) of DNA as template, 0.5 μl of each primer (5 μM), 2.0 μl of each dNTP (2 mM), 2.5 μl of 10× PCR buffer, 2.0 μl of (25 mmol l^{-1}) Mg^{2+} and 1 μl (1U μl^{-1}) of Taq DNA polymerase (Fermentas) and 15.5 μl sterile water. The PCR amplification profiles were as follows: 94°C initial denaturation for 4 min, 35 cycles of 94°C denaturation for 45 s, 58–63°C annealing for 45 s, and 72°C extension for 15–55 s (according the length of the target fragments), followed by a 10 min extension at 72°C. Information on primer sequences, restriction endonucleases and allele sizes were given in Supplemental Table 1 and Supplemental Table 2. For the PCR-RFLP assays, 8.5 μl of PCR products were digested with 5 U *Taq*I (*MC4R*), *Hin*fI (*LEP*) or *Hae*III (*H-FABP*) (Fermentas) in 1× digestion buffer added in a total volume of 10 μl, following digestion for 4 h at 65°C or 37°C. The restriction fragments of DNA were separated by electrophoresis in 2.5–4% agarose gel stained with ethidium bromide.

Multiplex PCR-RFLP

Multiplex PCR was performed in 25 μl of mix containing: 1 μl (50 ng) of DNA as template, 0.5 μl of each primer (*MC4R*-F and *MC4R*-R, *LEP*-F and *LEP*-R) (5 μM), 2.0 μl of each dNTP (2 mM), 2.5 μl of 10× PCR buffer, 2.0 μl of (25 mmol l^{-1}) Mg^{2+} and 1 μl (1U μl^{-1}) of Taq DNA polymerase and 14.5 μl sterile water. *MC4R* and *LEP* primers were given in Supplemental Table 1. The PCR reaction was carried out with the following cycling parameters: an initial 94°C for 4 min; 34 cycles of 94°C for 40 s, 59°C for 40 s, and 72°C for 40 s; and 72°C for 10 min. The mix of PCR products was digested with *Taq*I and *Hin*fI restriction endonucleases at 65 and 37°C, respectively, overnight. The reactions (final volume 10 μl) contained 1× digestion buffer, 5 U of restriction enzyme, 6 μl of PCR product and 2.5 μl of sterile water. The digested DNA was electrophoresed on 4% agarose gel containing ethidium bromide.

Statistical analysis

For association study, performance traits were recorded in 129 pigs of the DIV_2 population. The association between genotypes and traits recorded was performed with the general linear model (GLM) procedure (SAS Institute Inc., Cary, NC, USA). Both additive and dominance effects were estimated using the REG procedure. The additive effect was defined as −1, 0 and 1 for AA/AA/dd, AG/AB/Dd and GG/BB/DD (*MC4R*/*LEP*/*H-FABP*), respectively, and the dominance effect represented as 1, −1 and 1 for AA/AA/dd, AG/AB/Dd and GG/BB/DD (*MC4R*/*LEP*/*H-FABP*), respectively [13]. The statistical model was assumed to be: $Y_{ijk} = \mu + S_i + S_j + G_k + b_{ijk}X_{ijk} + e_{ijk}$, where Y_{ijk} is the observed values of traits; μ is the least-square mean; S_i is the effect of sex ($i = 1$ for male or 2 for female), S_j is the effect of season, G_k is the effect of genotype (k = AA/AA/dd, AG/AB/Dd and GG/BB/DD), b_{ijk} is the regression coefficient of the body weight, X_{ijk} is the body weight, and e_{ijk} is the random residual.

Linkage disequilibrium analysis

Maximum likelihood estimates of gametic frequencies and disequilibrium were obtained from genotypic counts [14, 15].

Results

Polymorphism detection

Two *MC4R* alleles were tested in the five populations under study: A (156 bp + 70 bp) and G (226 bp). Three genotypes, namely AA, AG and GG were observed. Sample size of each herd and allele frequencies are listed in Supplemental Table 3. The two Chinese indigenous breeds (Meishan and Bamei) contain predominantly the A allele but in different extension: A allele incidence ranges from 1 for Meishan to 0.6125 for Bamei. The gene frequencies in different western commercial pig breeds did not show the same trend. The frequency of A allele was 0.4286 in Yorkshire, 0.5077 in Landrace.

LEP genotypes of all the animals tested for *MC4R* are shown in Supplemental Table 4. Two different alleles of *LEP* gene were identified: alleles A (397 bp + 89 bp) and B (347 bp + 89 bp + 50 bp) that control the occurrence of three genotypes, namely AA, AB and BB. Genetic variation analysis results revealed that allele frequencies were significantly different between two Chinese indigenous breeds (Meishan and Bamei) and two western commercial pig breeds (Yorkshire and Landrace) and the Chinese indigenous breeds had higher frequencies of the B allele.

H-FABP genotypes of all populations identified for *MC4R* and *LEP* are summarized in Supplemental Table 5. At this locus, two alleles were identified under study: *D* (683 bp + 117 bp + 16 bp) and d (405 bp + 278 bp + 117 bp + 16 bp). Three genotypes, namely DD, Dd and dd were tested. Allele D was predominant in five pig populations, with the exception of Landrace and DIV_2 pigs, in which allele d was predominant.

Simultaneous amplification of fragments of *MC4R* and *LEP* gene

Multiplex PCR products were separated on a 1.5% agarose gel (Fig. 1). With *Taq*I restriction enzyme, the 486 bp PCR products of *LEP* gene were digested into two fragments of 354 bp and 132 bp. Fragments 156 and 70 bp were distinctive for the A allele and 226 bp fragment for G allele of the *MC4R* gene (Fig. 1). With *Hin*fI restriction enzyme, the 226 bp PCR products of *MC4R* gene were digested into two fragments of 141 bp and 85 bp. The presence of 397 and 89 bp fragments was characteristic for the A allele and 347, 89 and 50 bp for the B allele of the *LEP* gene (Fig. 1).

Statistical analysis

Association analysis was performed separately for each locus in DIV_2 line pigs. The data obtained showed that *MC4R*/*Taq*I polymorphism had significant associations with average backfat thickness and lean meat percentage. Furthermore, the average backfat thickness of pigs with AA genotype was significantly thinner than that of pigs with AG genotype ($P < 0.05$) and the lean meat percentage of pigs with AA genotype was significantly higher than that of pigs with AG genotype ($P < 0.05$) (Table 1). The results also indicated that there were additive effects on average backfat thickness and lean meat percentage (Unpublished data).

The results of the experiment indicated that *LEP*/*Hin*fI polymorphism had a significant association with the test daily gain. The test daily gain of pigs with AA genotype was significantly lower than those of pigs with BB genotype ($P < 0.01$) and AB genotype ($P < 0.05$), but no significant conclusion could be made on other economic traits (Table 2). At this locus, the additive effect seemed to be significant (Unpublished data).

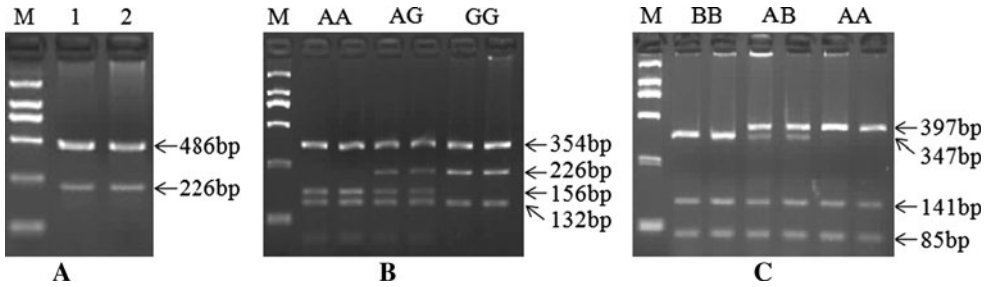

Fig. 1 Multiplex PCR-RFLP. **a** Simultaneous *MC4R-LEP* PCR results. 1–2: PCR products; **b** Discrimination of *MC4R* genotypes by digestion of the multiplex PCR products with *Taq*I. Genotypes are indicated on the top of the lanes. 354 bp and 132 bp are restriction fragment size of *LEP* PCR products; **c** Discrimination of *LEP* genotypes by digestion of the multiplex PCR products with *Hin*fI. Genotypes are indicated on the top of the lanes. 141 bp and 85 bp are restriction fragment size of *MC4R* PCR products. M: DNA Ladder

Table 1 Association between *MC4R* genotype and economic traits in DIV_2 line pigs

Traits	*MC4R* genotype ($\mu \pm$ SE)		*P* value		
	AA	AG	AA-AG	AA-GG	AG-GG
Average backfat thickness (mm)	18.15 ± 0.31	19.58 ± 0.48	0.0158	–	–
Lean meat percentage (%)	58.68 ± 0.33	57.46 ± 0.51	0.0492	–	–
Test daily gain (g)	811.73 ± 3.92	821.49 ± 6.03	0.1821	–	–
Feed conversion	2.85 ± 0.03	2.90 ± 0.04	0.3260	–	–

Table 2 Association between *LEP* genotype and economic traits in DIV_2 line pigs

Traits	*LEP* genotype ($\mu \pm$ SE)			*P* value		
	AA	AB	BB	AA-AB	AA-BB	AB-BB
Average backfat thickness (mm)	18.28 ± 0.40	18.76 ± 0.43	19.39 ± 0.66	0.4070	0.1504	0.4213
Lean meat percentage (%)	58.71 ± 0.42	58.00 ± 0.45	57.60 ± 0.69	0.2523	0.1745	0.6259
Test daily gain (g)	807.45 ± 4.78	821.60 ± 5.07	832.87 ± 7.83	0.0449	0.0069	0.2301
Feed conversion	2.84 ± 0.03	2.90 ± 0.04	2.87 ± 0.06	0.2884	0.7068	0.6721

Table 3 Association between *H-FABP* genotype and economic traits in DIV_2 line pigs

Traits	*H-FABP* genotype ($\mu \pm$ SE)			*P* value		
	DD	Dd	dd	DD-Dd	DD-dd	Dd-dd
Average backfat thickness (mm)	17.29 ± 1.14	18.86 ± 0.38	19.29 ± 0.43	0.1905	0.1044	0.4677
Lean meat percentage (%)	60.18 ± 1.12	58.11 ± 0.38	57.62 ± 0.43	0.0826	0.0362	0.3973
Test daily gain (g)	803.06 ± 13.94	817.93 ± 4.71	817.47 ± 5.28	0.3142	0.3384	0.9491
Feed conversion	2.90 ± 0.09	2.89 ± 0.03	2.85 ± 0.03	0.9264	0.6176	0.3888

The *H-FABP*/*Hae*III polymorphism was associated with lean meat percentage but not other economic traits. Pigs carrying genotype DD had higher lean meat percentage than the dd individuals ($P < 0.05$) (Table 3).

Linkage disequilibrium analysis

The gametic frequencies of the joint study of *MC4R* and *LEP* loci were listed in Table 4. Different dispersion values were observed for the different populations. Two gametic types were found in Meishan (gamete AA & gamete AB). For other populations, all of the four gametic types were revealed.

Gametic frequencies of the joint study of *MC4R* and *H-FABP* loci were reported in Table 5. In Meishan pigs, 100% of the animals were gamete AD. Gametic types of the highest incidence were Ad, GD, Ad and AD in DIV_2, Yorkshire, Landrace and Bamei, respectively.

For *LEP* and *H-FABP* loci, different gametic structures were observed between Chinese breeds and Western commercial pig breeds (Table 6). Gamete BD showed the highest incidence in Meishan and Bamei. Gametic types of the lowest frequency were BD, Bd and BD in DIV_2, Yorkshire and Landrace, respectively.

The linkage disequilibrium between two loci (*MC4R*& *LEP*, *MC4R*&*H-FABP* and *LEP*&*H-FABP*) across all populations was not significant ($P > 0.05$) (Tables 4, 5, and 6). Results revealed that there was not an association between both loci. Therefore, exerting selection pressure on one locus should not influence the allelic frequencies of the other locus.

Discussion

We detected the mutation of the *MC4R* gene in different populations. The observed allele A frequencies in our sample were 0.4286 and 0.5077 for Yorkshire and Landrace, respectively. A lower frequency of allele A was observed in Polish Large White[16]. A higher frequency of the allele A compared with that in the present study was revealed in Polish Landrace pigs [16]. It was similar to the finding of Chen et al. [17] that Allele A was dominant in Bamei.

Table 4 Gametic, gene frequencies and overall disequilibrium in the five studied populations for the *MC4R* and *LEP* loci

	DIV_2	Yorkshire	Landrace	Meishan	Bamei
Gametic type					
$MC4R^{A}LEP^{A}$	0.564	0.380	0.469	0.400	0.138
$MC4R^{A}LEP^{B}$	0.309	0.048	0.038	0.600	0.475
$MC4R^{G}LEP^{A}$	0.084	0.497	0.462	0.000	0.138
$MC4R^{G}LEP^{B}$	0.043	0.075	0.031	0.000	0.250
Allele					
$MC4R^{A}$	0.873	0.429	0.508	1.000	0.613
LEP^{B}	0.352	0.123	0.069	0.600	0.725
Disequilibrium					
d	0.002*	0.004*	0.003*	–	0.031*

* $P > 0.05$

Table 5 Gametic, gene frequencies and overall disequilibrium in the five studied populations for the *MC4R* and *H-FABP* loci

	DIV_2	Yorkshire	Landrace	Meishan	Bamei
Gametic type					
$MC4R^{A}$H-$FABP^{D}$	0.291	0.393	0.150	1.000	0.582
$MC4R^{A}$H-$FABP^{d}$	0.582	0.036	0.358	0.000	0.031
$MC4R^{G}$H-$FABP^{D}$	0.044	0.510	0.188	0.000	0.356
$MC4R^{G}$H-$FABP^{d}$	0.083	0.061	0.304	0.000	0.031
Allele					
$MC4R^{A}$	0.873	0.429	0.508	1.000	0.613
H-$FABP^{D}$	0.335	0.903	0.339	1.000	0.938
Disequilibrium					
d	0.001*	0.006*	0.022*	–	0.007*

* $P > 0.05$

Table 6 Gametic, gene frequencies and overall disequilibrium in the five studied populations for the *LEP* and *H-FABP* loci

	DIV_2	Yorkshire	Landrace	Meishan	Bamei
Gametic type					
LEP^{A}H-$FABP^{D}$	0.218	0.786	0.327	0.400	0.263
LEP^{A}H-$FABP^{d}$	0.431	0.091	0.604	0.000	0.012
LEP^{B}H-$FABP^{D}$	0.117	0.117	0.011	0.600	0.675
LEP^{B}H-$FABP^{d}$	0.234	0.006	0.058	0.000	0.050
Allele					
LEP^{B}	0.352	0.123	0.069	0.600	0.725
H-$FABP^{D}$	0.335	0.903	0.339	1.000	0.938
Disequilibrium					
d	0.001*	0.006*	0.012*	–	0.005*

* $P > 0.05$

The *MC4R* gene had been assigned to porcine chromosome 1 (SSC1). SSC1 encompassed the largest number of QTLs that were economically important in pig breeding. Karlskov-Mortensen et al. [18] demonstrated that the *MC4R* gene was located in the QTL affecting fat/meat content on the carcass in a Hampshire-Landrace population. Statistically significant associations with economic traits were found for *MC4R*/*Taq*I polymorphism that was in the coding region of the *MC4R* gene [4]. Houston et al. [3] reported that allele A was associated with low backfat and slow growth rate in large white pig populations, while allele G was associated with faster-growing and fatter animals. Similarly, studies on Lithuanian White pigs revealed the mutant Asn298 allele of the *MC4R* gene was significantly associated with increased test daily gain, higher lean meat percentage and lower backfat thickness [19]. In contrast, Park et al. [20] found no significant association between the *MC4R* polymorphism and fatness

related traits in a Large White × Wild Boar intercross F_2 population. Based on studies performed with several breeds and lines of pigs, the significance of the effect of genotype at the *MC4R* locus on growth and fatness may vary depending on breed or line. In addition, the single missense mutation (Asp298Asn) of aspartic acid (Asp) to asparagine (Asn) in *MC4R* gene decreased cAMP content and MC4R signaling [21]. This finding will undoubtedly help in further understanding the relationship between *MC4R*/*Taq*I polymorphism and meat quality.

The analysis of *LEP* gene polymorphism showed that the allele A was predominant in western commercial pig breeds such as Yorkshire, Landrace. It was similar to the finding of Kennes et al. [22] who studied the Yorkshire and Landrace. Synthetic Line DIV_2 was a result of cross of Yorkshire, Landrace, Meishan and/or Tongcheng pigs, and had more genetic basis of Yorkshire and Landrace pigs, thereby, the increased A allele incidence.

The SSC18 chromosome had the fewest markers and the greatest average interval between markers of any porcine chromosome. Increasing the marker density would facilitate understanding of the structural organization of this chromosome. The analysis of the Wild boar × Meishan family indicated that the QTLs for carcass leanness and fat-to-meat ratio were localized near the *LEP* gene [23]. Our results showed an association between the B allele and high test daily gain. de Oliveira Peixoto et al. [8] reported the effect of leptin genotype on production traits in a F_2 population and they showed that allele A was associated with high weights at 21, 42, 63 and 77 days of age. However, the average daily gain and feed conversion from 77 to 105 days of age of pigs with AB genotype were significantly higher than that of pigs with AA genotype. Previously, Kennes et al. [22] found association between T3469C polymorphism and the average daily weight gain in Landrace. However, Szydlowski et al. [24] found no major effect of the leptin gene polymorphism (T3469C) on growth and carcass traits in Polish Large White, Polish Landrace and a synthetic pig line. The inconsistency may be partly due to differences in the genetic background of the analyzed breeds. Moreover, the T3469C polymorphism does not affect the encoded protein structure. It means that a direct effect of this polymorphism is unlikely.

In our research, genetic variation analysis of *H-FABP* gene polymorphism revealed that the gene frequency of Chinese indigenous breeds with *Hae*III-RFLP was similar to other reports [25, 26]. The genotypes showed a diverse distribution in western commercial pig breeds for the intron 2 specific RFLPs.

Several studies in different populations had reported QTLs affecting intramuscular fat (IMF) and other traits on porcine chromosome 6 [27, 28]. The *H-FABP* gene was considered candidate gene for IMF due to its position and physiological role [29]. The *H-FABP* gene has been sequenced and three polymorphisms were detected, one in the upstream region (*Hin*fI) and two in the second intron (*Hae*III and *Msp*I) [10]. Previous studies focused on the relationship between *H-FABP* gene and IMF. For example, in the experiment with Duroc populations, the dd genotype (*Hae*III RFLP) showed increased IMF content and the DD (*Hae*III RFLP) genotype class had decreased backfat at a standard weight of 110 kg [11]. The results reported in the Meishan crossbred population indicated that *H-FABP* can be used to affect IMF content without an effect on backfat thickness [27]. Furthermore, Pang et al. [30] demonstrated that fat deposition in adipocytes was stronger in the dd genotype than in the DD genotype. Results of the present study showed that the *H-FABP* RFLP polymorphism (*Hae*III) had a significant association with lean meat percentage in DIV_2 population. Selection for leanness of porcine carcasses in the past has resulted in some reductions in IMF content mainly because of a mild but positive correlation with backfat thickness. There is a need to explore possibilities to increase the IMF content in lean meat while continuing selection for leaner carcass.

We developed a multiplex PCR to amplify *MC4R* and *LEP* gene simultaneously. The results obtained by multiplex PCR–RFLP were in 100% accordance with the ones obtained by "classical" methods. Moreover, the cost of the sample analysis will be decreased, and the speed and efficiency will be increased. Therefore, the method described in this paper could be widely utilized in breeding programs.

The linkage disequilibrium analysis between alleles at different loci indicated that three genes (*MC4R*, *LEP2*, *H-FABP*) in different chromosomes are independent. This allows the use of multiple genes associated with economic traits in pig for marker-assisted selection (MAS) systems. MAS can be used to pyramid several genes into individuals that are homozygous at all loci. For pig breeders, one advantage of multiple marker assisted selection is that it provides opportunities to enhance response to selection, in particular for traits that are difficult to improve by conventional selection. For example, production of lean carcasses and meat would require selection of *MC4R* AA genotypes and *H-FABP* DD genotypes in DIV_2 line. Another advantage is simultaneous selection for multiple traits. For example, increased lean meat percentage and test daily gain would require selection of *MC4R* AA genotypes and *LEP* BB genotypes in DIV_2 line. Furthermore, simultaneous selection for multiple traits is a breeding strategy, which with the inclusion of multiple marker assisted selection can shift undesirable correlations.

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