Genetic variation at *RYR1*, *IGF2*, *FUT1*, *MUC13*, and *KPL2* mutations affecting production traits in Chinese commercial pig breeds

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ABSTRACT: The identification of causative mutations affecting economically important traits has benefited the worldwide pig industry. We investigated the genetic variation at five loci including *RYR1*, *IGF2*, *FUT1*, *MUC13*, and *KPL2* affecting traits related to production, reproduction, and disease resistance in a sample of 8009 pigs representing 3 commercial breeds (Duroc, Landrace, and Large White) from 28 farms in China. We found that all breeds, especially Duroc pigs, have high frequencies of favourable alleles for lean production and stress resistance at the *IGF2* and *RYR1* loci. However, all breeds have low frequencies of the diarrhea-resistant allele of *FUT1*, indicating that multigenerational selection is required for *E. coli* F18$^+$ resistant pigs. No linkage disequilibrium was found between the *RYR1* and *FUT1* loci on pig chromosome 6, supporting the possibility of combined selection for both F18 and stress-resistant pigs. Relatively high frequencies (> 0.5) of the *MUC13* allele conferring resistance to *E. coli* F4ac were found in all three breeds with the highest frequency in Duroc pigs, suggesting that the breeders can establish F4ac diarrhea-resistant lines in a few generations. No defective allele at the *KPL2* locus causing immotile short-tail sperms was found in Large White pigs of American, Canadian, Danish, English, and French origin, supporting the conclusion that the *KPL2* defective allele is present exclusively in Finnish Large White pigs. These results provide useful information for pig breeding schemes in China.

Keywords: China; commercial pig breed; genetic variation; economically important markers

Production, reproduction, and disease resistance traits are economically important for the pig industry. Genetic improvement of these traits can enhance the swine profitability and growth performance. Up to now, causative mutations for these economically important traits have been identified in a limited list of genes in pigs, such as *RYR1*, *IGF2*, *FUT1*, *MUC13*, and *KPL2*. Breeders have integrated these important variants into their breeding programs both in China and western countries.

The *RYR1* c.1843C>T mutation causes malignant hyperthermia and pale, soft and exudative (PSE) pork (Fujii et al., 1991). The PSE pork is regarded as inferior quality meat and has caused huge economic loss in the pig industry. The *IGF2* intron3 g.3072G>A substitution has been explicitly identified as a quantitative trait nucleotide and explains a paternally-expressed imprinting QTL (Jeon et al., 1999; Nezer et al., 1999) for muscle growth, fat deposition, and heart size (Van Laere et al., 2003). Several reports have confirmed that

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pigs carrying the paternal *A* allele have higher lean growth and lower backfat thickness compared with those having the maternal *G* allele (Jungerius et al., 2004; Estellé et al., 2005; Oczkowicz et al., 2009). Diarrhea is a major enteric disease in piglets, resulting in higher mortality and treatment costs. In some cases, morbidity exceeds 80% and mortality is higher than 25% (Gregory and Grandin, 2007). Enterotoxigenic *Escherichia coli* (ETEC) F4ac and F18 are two common pathogens causing diarrhea. Meijerink et al. (2000) reported that the *FUT1* c.307G>A mutation affects susceptibility to ETEC F18-caused diarrhea in Large White and Landrace pigs. By a battery of genetic analyses, Ren et al. (2012) showed the compelling evidence that *MUC13* is a good candidate gene encoding the F4ac intestinal receptor. A significant marker (g.28784 T>C) accurately distinguishing susceptible and resistant animals has been identified in this gene. In 1987, an immotile short-tail sperm (ISTS) defect was first found in Finnish Large White pigs. The defect has been shown to be caused by an intronic 9 kb insertion in the *KPL2* gene (Sironen et al., 2006). We herein investigated the genetic variation of the five loci of *RYR1*, *IGF2*, *FUT1*, *MUC13*, and *KPL2* in a large sample of nucleus herds in China, providing fundamental information for Chinese pig breeding schemes.

MATERIAL AND METHODS

Animals

A total of 8009 animals were collected from 72 nucleus herds of 28 pig breeding farms in 20 provinces of China, including 2961 Duroc pigs (459 males and 2502 females), 1860 Landrace pigs (284 males and 1576 females), 3005 Large White pigs (334 males and 2671 females), and 183 Synthetic Line pigs (17 males and 166 females). Pig ear tissues were collected and genomic DNA was extracted by using phenol/chloroform extraction and ethanol precipitation.

Genotyping

Primers (Table 1) were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/). PCR reactions were performed in 20 µl volume containing 1 × PCR buffer, 1.5mM $MgCl_2$, 150µM of each dNTP, 0.2µM of each primer, 40 ng of genomic DNA, and 2.5 units of *Taq* polymerase (Shenergy Biocolor BioScience, Shanghai, P.R. China). The amplifications were carried out in PTC-200 thermocyclers (Bio-Rad, Waltham, USA) with an initial denaturation at 94°C for 3 min, 36 cycles of 30 s at 94°C, 45 s at optimal annealing

Table 1. PCR primers and amplicons of *IGF2*, *RYR1*, *FUT1*, *MUC13*, and *KPL2* genes

Locus	Primer (5'→3')	Amplicon (bp)	Temperature (°C)	Genotyping method
IGF2 intron3 g.3072 G>A	F: ACTGTTGAAGTCCCCGAGAG R: GAAGGGAGGAAGCCGAGAG SNaPshot: TTTTTTTTTTCCGGGCCGCGGCTTCGCCTAGGCTC	283	66	SNaPshot
RYR1 c.1843 C>T	F: TCCAGTTTGCCACAGGTCCTACCA R: ATTCACCGGAGTGGAGTCTCTGAG	659	64	*Hha*I PCR-RFLP*C*: 493 bp + 166 bp *T*: 659 bp
FUT1 c.307 G>A	F: CTTCAGCCAGGGCTCCTTTAAG R: CTG CCT GAA CGT CTA TCA AGA CC	421	56	*Hin*6I PCR-RFLP *G*: 241 bp + 93 bp + 87 bp *A*: 328 bp + 93 bp
MUC13	F: GGAGAGACCAAACCCACAGA R: CTCCTCACCAGCTCCTTAGC SNaPshot: TTTTTTTTTTTTTTTCCATGTACATTTCAGAGTCTGAGGGAT	280	61	SNaPshot
KPL2	F: GGCAATATCAAGGTCTTTCCA R1: GCAGGAGAGGAGAATGACCA R2: GTGCCCGTAGTTCAGATGGT	354 709	64	PCR *ins/ins*: 709 bp *-/ins*: 354 bp + 709 bp *-/-*: 354 bp

temperatures (Table 1), and 45 s at 72°C, followed by a final extension at 72°C for 10 min.

The *RYR1* c.1843C>T mutation was genotyped by *Hha*I PCR-RFLP with digestion of 6 µl PCR product using 1 unit of *Hha*I (New England Biolabs, Hercules, USA) at 37°C for 4 h. The digested products were separated in 2.0% agarose gels for genotype recording (Table 1). *Hin*6I PCR-RFLP was used to genotype the *FUT1* c.307G>A mutation as described previously (Yan et al., 2003; Table 1). The *KPL2* insertion was detected by a PCR-based test using primers listed in Table 1 as described previously (Sironen et al., 2007). The test can identify both hetero- and homozygous carriers of the mutation (Table 1).

The *IGF2* intron3 g.3072G>A and *MUC13* loci were genotyped with the SNaPshot Multiplex Kit (Applied Biosystems, Foster City, USA). PCR products of 1.5 µl were purified with 0.3 units of Shrimp Alkaline Phosphatase (SAP) and 0.2 units of Exonuclease I. The mixture was incubated at 37°C for 40 min and then followed by an incubation at 75°C for 15 min. Subsequently, the SNaPshot PCR was implemented in a 5 µl volume containing 1.5 µl of purified PCR products, 2 µl of SNaPshot Multiplex Kit, and 0.25 µl of each SNaPshot primer (10 pmol/µl) (Table 1). The reaction program consisted of 40 (*IGF2*) or 25 (*MUC13*) cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s. Then the SNaPshot PCR product was purified with 1 unit SAP at 37°C for 1 h and followed by a step of 75°C for 15 min. To the end, 1 µl of the purified SNaPshot PCR product was run in an ABI PRISM 3100xl Genetic Analyzer (Applied Biosystems, Foster City, USA). Genotypes were finally recorded with GeneMapper® Software (Version 4.1, 2000).

Statistical analysis

The allele frequencies and their deviation from Hardy-Weinberg equilibrium (HWE), pairwise linkage disequilibrium values r^2 and D' were calculated by the GENEPOP (Version 3.3, 2002) computer package (Raymond and Rousset, 1995).

RESULTS AND DISCUSSION

The allele and genotype frequencies as well as the deviation from HWE at the tested mutation sites are presented in Table 2.

RYR1. The desirable allele (*C*) at the *RYR1* c.1843C>T locus conferring resistance to malignant hyperthermia was fixed in two Synthetic Lines and it was predominant in Duroc (0.917), Large White (0.981), and Landrace (0.972) pigs (Table 2). After the identification of the *RYR1* c.1843C>T mutation in 1991 (Fujii et al., 1991) many breeding companies have used the DNA diagnostic test to eliminate homozygous stress positive (*TT*) individuals and heterozygous carriers (*TC*). Therefore, the high frequency of the *C* allele can be explained by selection. It is noteworthy that a number of heterozygotes and several undesirable *TT* homozygotes were found in Duroc, Large White, and Landrace breeds. This clearly indicates that Chinese breeders should keep a continuous selection on the *RYR* locus to eventually establish malignant hyperthermia-free lines and populations.

IGF2. At the *IGF2* intron3 g.3072 G>A locus, the favourable *A* allele increasing lean production was almost fixed in Duroc pigs and it was present at high frequencies in Landrace and Large White pigs. Duroc pigs have the highest frequency of 0.932, followed by 0.756, 0.767, and 0.788 in Landrace, Large White, and Synthetic Line 2 pigs, respectively (Table 2). The result is in accordance with the data from Ojeda et al. (2008) and Yang et al. (2006). It is most likely caused by long-term selective breeding for lean production in Duroc, Landrace, and Large White pigs. The intensive selection caused the significant deviation from HWE ($P < 0.01$) at the *IGF2* locus in these breeds. Recent data showed that the *A* allele at this locus is significantly associated with low sow reproduction performance (Stinckens et al., 2010). As *IGF2* is a paternally-expressed imprinting gene (Van Laere et al., 2003), it has been suggested to select *AA* boars in sire lines and *GG* animals in hybrid sows for increasing both sow productivity and lean percentage (Stinckens et al., 2010).

FUT1. At the *FUT1* c.307G>A locus, Duroc, Landrace, and Large White pigs have low frequencies of the *A* allele that is associated with resistance to F18ab, with values of 0.278, 0.061, and 0.092, respectively (Table 2). Of the three breeds, only Landrace exhibited significant deviation from HWE at this mutation site ($P < 0.01$). Previous reports also showed low frequencies of resistant animals in pigs from western countries. Meijerink et al. (1997) reported that no more than 5–10% of Swiss Landrace, Large White, and Duroc pigs are resistant to F18. In Belgian hybrid pigs, only a

Table 2. Allele frequencies and the deviation from Hardy-Weinberg equilibrium (HWE) at the tested mutation sites

Locus	No. of animals	Allele frequency		HWE (*P*-value)
***IGF2* intron3 g.3072G>A**[a]		A	G	
Duroc	561	0.932	0.068	9.3E-36
Landrace	1602	0.756	0.244	2.4E-19
Large White	2636	0.767	0.233	4.6E-11
Synthetic Line 2	113	0.788	0.212	0.778
***RYR1* c.1843C>T**[b]		C	T	
Duroc	1010	0.917	0.083	0.402
Large White	1028	0.981	0.019	0.048
Landrace	2071	0.972	0.028	0.019
Synthetic Line 1	55	1.000	0.000	1.000
Synthetic Line 2	126	1.000	0.000	1.000
***FUT1* c.307G>A**[c]		A	G	
Duroc	205	0.278	0.722	0.728
Large White	431	0.061	0.939	0.210
Landrace	794	0.092	0.908	2E-08
MUC13[d]		T	C	
Duroc	2828	0.210	0.790	0.090
Landrace	1329	0.438	0.562	0.001
Large White	2853	0.503	0.497	0.940
Synthetic Line 1	55	0.427	0.573	0.001
Synthetic Line 2	126	0.623	0.377	0.731
KPL2[e]		M	m	
Large White	2987	1.000	0.000	1.000

[a]allele *A* is favourable allele for lean production and allele *G* is unfavourable allele

[b]allele *C* is malignant hyperthermia resistant allele and allele *T* is susceptible allele

[c]allele *A* is desirable allele conferring resistance to ETEC F18 and allele *G* is susceptible allele to ETEC F18

[d]*C* is associated with resistant allele and *T* is associated with susceptible allele; piglets with *TT* and *TC* genotypes are associated with diarrhea-susceptible animals, while *CC* piglets are associated with diarrhea-resistant animals (Ren et al., 2012)

[e]*M* is wild-type allele and *m* is mutant allele causing immotile short-tail sperms

small fraction (4.6%) of animals is resistant to F18 infection (Coddens et al., 2008). In this study, 8.3% of Duroc pigs are homozygous resistant animals, while smaller proportions of resistant animals were found in Large White (0.7%) and Landrace (2.5%) pigs. Hence, a long-term breeding program is required to select for F18 resistant pig lines. From 1999 to 2005, PIC company selected for the resistant allele of *FUT1* and increased the proportion of resistant animals from 8 to 35% in commercial pigs (van der Steen et al., 2005).

Linkage disequilibrium between *FUT1* and *RYR1*. Both *FUT1* and *RYR1* map to pig chromosome 6. Meijerink et al. (1997) reported strong linkage disequilibrium between the two loci by showing a 93% association between *FUT1 A* (favourable) and *RYR1 T* (unfavourable) alleles in Swiss Landrace pigs. In contrast, no linkage disequilibrium was found between the *FUT1* and *RYR1* loci in a Belgium pig population (Coddens et al., 2008). We herein found very low r^2 values between the two loci in Duroc, Landrace, and Large White pigs (Table 3). This result is consistent with the report by Coddens et al. (2008), allowing the possibility of combined selection for both F18 and stress-resistant pigs.

Table 3. Linkage disequilibrium between the *FUT1* and *RYR1* loci in Duroc, Landrace, and Large White pigs

Breed	No.	Disequilibrium	
		D'	r^2
Duroc	198	0.394	0.133
Large White	758	0.039	0.001
Landrace	411	0.023	0.001

Table 4. Percentage of animals with *IGF2*, *RYR1*, and *MUC13* favourable genotypes in different pig populations[a]

Farm	Breed	No.	Genotype (%)			
			IGF2 AA	*RYR1 CC*	*MUC13 GG*	*AA/CC/GG*
1	Duroc	211	68.4	86.7	41.3	33.1
2	Duroc	211	95.0	100	37.9	37.4
3	Landrace	108	61.1	100	34.6	19.5
4	Large White	158	91.8	100	31.6	29.1
5	Large White	99	67.7	93.9	34.3	25.3
6	Large White	284	91.8	100	31.6	29.1
7	Large White	112	54.5	88.4	32.6	22.7
8	Large White	190	71.1	100	39.2	18.7
9	Synthetic Line	111	62.2	100	28.7	19.0

[a]animals from 9 pig farms in 8 Chinese provinces

MUC13. We have recently demonstrated that *MUC13* is a good candidate gene encoding the intestinal ETEC F4ac receptor in pigs (Ren et al., 2012). In this study, we genotyped a *MUC13* SNP accurately distinguishing pigs susceptible or resistant to ETEC F4ac. In contrast to the *FUT1* result, we found relatively high frequencies of the resistant allele in Duroc (0.790), Landrace (0.562), Large White (0.497), and Synthetic Line 1 pigs (0.573) (Table 2). Notably, Duroc pigs have a much higher frequency of the resistant allele at this locus. This is in agreement with our previous report that a higher percentage of F4ac-resistant animals were observed in Duroc pigs compared with Landrace and Large White pigs (Yan et al., 2009). It allows pig breeders to generate F4ac-resistant Duroc lines in few generations by selecting for the resistant allele.

KPL2. The *KPL2* insertion causing ISTS was originally identified in Finnish Large White pigs. In this study, we did not find the defective insertion at the *KPL2* locus in 2987 Large White pigs of American, Canadian, Danish, English, and French origin, as all samples had the single amplicon of 354 bp (Table 2). The result indicates that Chinese commercial pigs have not been affected by the genetic defect, and supports the conclusion that the *KPL2* defective insertion was present exclusively in Finnish Large White pigs (Sironen et al., 2007).

Animals with favourable genotypes. To evaluate the feasibility of multi-locus selection, we analyzed the percentages of pigs with the favourable genotypes (*AA/CC/GG*) at the *IGF2*, *RYR1*, and *MUC13* loci. The *KPL2* and *FUT1* loci were discarded for this analysis because of the fixation of the *KPL2* wild-type allele and very low frequencies of the *FUT1* favourable allele in the tested populations. We found that the percentage values ranged from 19.0 to 37.4% in the populations (Table 4). Only two Duroc populations had more than one third of animals carrying the desirable *AA/CC/GG* genotypes. The observation indicates that multigenerational selection is required to fix the favourable alleles at the three loci in Chinese commercial breeds.

In conclusion, we genotyped a large number of animals representing Duroc, Landrace, and Large White breeds from 72 nucleus farms of 20 Chinese provinces. The large-scale investigation provides significant information for breeding schemes and would benefit the pig industry in China.

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