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Identification and characterization of *RFRP* gene in pigs and its association with reproductive traits

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ABSTRACT. RFamide-related peptide (*RFRP*) gene is a crucial gene of the hypothalamo-pituitary-gonadal axis and plays an important role in reproduction regulation. This study aimed to clone and characterize the pig *RFRP* gene. We obtained a 645-bp cDNA of pig *RFRP* gene comprising a 546-bp open reading frame, which encoded a peptide of 188 amino acids. The pig RFRP coding sequences have the identities of 81, 68.8, and 76.1% with their counterparts in humans, mice, and rats, respectively. Real-time polymerase chain reaction showed that the pig *RFRP* gene was expressed predominantly in the stomach and testis of males, and large intestine and uterus of females. It was also found

to be abundantly expressed in the cerebrum and liver tissues of both male and female pigs. The pig *RFRP* gene is 4.6 kb long and contains 3 exons and 2 introns. We further identified a total of 25 single nucleotide polymorphisms and 2 insertion/deletion (indel) polymorphisms across the whole *RFRP* gene, and 9 of them were in the exons. Marker trait association analysis in Yorkshire and Landrace populations showed that g.45859759C>T was significantly associated with the total number born of second parity (TBA2; $P < 0.05$). In conclusion, the pig *RFRP* gene was cloned and characterized in this study, and its polymorphism g.45859759C>T showed significant associations with reproductive traits.

Key words: Pig; *RFRP* gene; SNP; Reproductive trait; Association analysis

INTRODUCTION

After its isolation from *Macroeallista nimboia* in 1977, FMRFamide was found to stimulate the heart and inhibit food intake (Price and Greenberg, 1977). Subsequently, a variety of peptides with an RFamide structure at their C terminus have been identified. Thus far, 5 types of peptides, including KISS, LPXRFa, QFRP, PQRFa, and PrRP, with the same RFamide structure have been identified from birds and mammals (Kriegsfeld, 2006; Ebling and Luckman, 2008). The RFamide-related peptide (RFRP) gene, a member of the RFamide family and an ortholog to the gonadotropin-inhibiting hormone (GnIH) gene, was first detected in quail and found to encode 3 biological peptides, RFRP-1, RFRP-2, and RFRP-3 (Hinuma et al., 2000; Legagneux et al., 2009). In mammals, however, the RFRP gene only encodes 2 isoforms, RFRP-1 and RFRP-3.

The human *RFRP* gene at chromosome 7p21-p15 consists of 3 exons and 2 introns and encodes 2 major peptides, RFRP-1 and RFRP-3 (Hinuma et al., 2000). The function of *RFRP* gene is well studied in the nervous system, but limited in other peripheral systems. Hypothalamo-pituitary-gonadal is considered as a crucial axis that regulates reproductive activity through the secreted hormones (Kriegsfeld et al., 2010). Unlike the other 5 types of peptides, KISSpeptin has a more remarkable impact on puberty (Seminara et al., 2003). Until recently, RFRP was found to promote gonadotropin secretion via gonadotrophin releasing hormone (GnRH) neurons (Yoshida et al., 2003; Ubuka et al., 2009). Accordingly, RFRP peptide might play a key role in the seasonal activity of reproduction dependent on melatonin. The level of RFRP mRNA was found to be reduced to a greater extent to facilitate adaption to the short-day photoperiod (SD) than to long-day photoperiod (LD; Revel et al., 2008). The RFRP peptide was indicated to have an important impact on food intake (Bechtold and Luckman, 2007; Clarke et al., 2009). Furthermore, RFRP-1 peptide might help the function of cardiac contractility and might be a target for future drug development (Nichols et al., 2010). Recently, RFRP-3 was found to inhibit the release and synthesis of GnRH, gonadotrophin, and steroid hormones and impact the expression of KISS1, GnRHR, cyclin B1, PCNA, and ERK 1/2 (Li et al., 2013).

Therefore, the RFRP gene was thought to be a candidate gene for reproductive traits and energy balance. In pigs, the *RFRP* gene has not yet been identified; therefore, we aimed to clone its cDNA to investigate its expression among various tissues and between males and females, as well as to reveal the associations of *RFRP* gene polymorphisms with reproductive traits.

MATERIAL AND METHODS

Animals

Two 120-day-old Yorkshire pigs (one female and one male) were slaughtered to collect 40 tissues (female, 21 and male, 19), i.e., cerebrum, cerebellum, hypothalamus, pituitary, heart, liver, spleen, lung, kidney, subcutaneous fat, abdominal fat, foreleg muscle, back leg muscle, back muscle, small intestine, large intestine, stomach, ovary, oviduct, uterus, and testicle. Only ovary and uterus were used to clone the pig *RFRP* gene, and all tissues were used for real-time quantitative polymerase chain reaction (qPCR) analyses. Five females each of Yorkshire, Landrace, Duroc, Lantang, and Small-ear spotted pigs were used for the identification of polymorphisms across the complete coding region of the pig *RFRP* gene. Meanwhile, Yorkshire (sample size = 334) and Landrace (187) populations were also used for marker trait association analyses. The detail record of total number born (TNB) and number born alive (NBA) from first to sixth parity of each female of these 2 populations was available. Small pieces of ears of all pigs were collected for the extraction of genomic DNA. These samples were provided by Guangdong Wens Foodstuff Corporation Ltd. (Guangdong, China) and Yunnan Province, China.

Primers

A total of 15 primers were used in this study (Table 1). Primers P1 and P2 for cDNA cloning were designed on the basis of the predicted RFRP cDNA sequences. P3 and P4 for pig *RFRP* and β-actin genes each were used for qPCR analyses. P5-P10 were used for the identification of *RFRP* gene polymorphisms according to the pig genome database. P8 and P11-P14 were designed to genotype 5 single nucleotide polymorphisms (SNPs) by the PCR-RFLP method. All primers were designed using the GeneTool Lite software (http://www.BioTools.com/) and then synthesized by GeneRay Co. Ltd. (Shanghai, China).

DNA extraction and RNA isolation

Total RNA of Yorkshire (21 females and 1 male) pigs was extracted from fresh or frozen tissues by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using 2 μg total RNA by reverse transcription using PrimeScript RT reagent Kit (TaKaRa, China). Genomic DNA was extracted from pig ears by using the typical phenol-chloroform method (Sambrook et al., 1989).

Cloning of pig RFRP cDNA

RT-PCR was performed to amplify the RFRP cDNA from the ovary and uterus by using primers P1 and P2. The 50-μL reaction mixtures contained 2 μL cDNA, 1 μL primers, 5 μL 10X TransTaq HiFi buffer, 4 μL 2.5 mM dNTPs, 0.5 μL TransTaq HiFi DNA polymerase, and distilled water. PCR was performed in a Mastercycler gradient (Eppendorf Limited, Hamburg, Germany) by using the following procedure: 94°C predenaturation for 3 min, followed by 35 cycles of 30 s at 94°C, 30 s at 64°C, and 45 s at 72°C, and a final extension of 8 min at 72°C. PCR products were checked on 1.5% agarose gels, and then purified using the Gel Extraction Kit (U-gene, Chi-

na). Subsequently, PCR products were cloned into the pMD18-T Easy plasmid vector (Promega, USA), and then sequenced by BGI-Shenzhen (Guangdong, China) with commercial service.

Table 1. Primers used in this study.

Gene	Primer	Sequence (5'-3')	AT (°C)	Product size (bp)	Purpose
RFRP	PM1	F: 5'-gctgcacacagaaacttt-3' R: 5'acttctgcatcatctattttcttg-3'	64	601	cDNA cloning by RT-PCR
	PM2	F: 5'-atgggaaaaacaaaggagtctca-3' R: 5'-aggctctggagttcttatttttc-3'	64	546	
	PM3	F: 5'-tcccaatctgccccaaag-3' R: 5'-ccctttggcagatggtgaa-3'	60	103	Real-time PCR
β-actin	PM4	F: 5'-ccgtgagaagatgacccagat-3' R: 5'-gccagccaggtccagacgc-3'	60	202	
RFRP	PM5	F: 5'-acggggatggatctcactgc-3' R: 5'-tgggcctagtctggggttaata-3'	64	785	Polymorphism in exon 1
	PM6	F: 5'-gcccatcccacttcctc-3' R: 5'-tcccactgtctttccatgtc-3'	62	694	PolymorphiSm in intron 1
	PM7	F: 5'-tggaaagacagtgggagacatc-3' R: 5'-gcgggtgatggagtaaagtaac-3'	62	764	Polymorphism in exon 2 and PCR-RFLP
	PM8	F: 5'-cctccacacttgcaccactga-3' R: 5'-ctgcggcaatgctggatc-3'	63	878	Polymorphism in intron 2
	PM9	F: 5'-atggctcctagtcagatttgttt-3' R: 5'-acctcagcagaaacgtcagtg-3'	55	864	Polymorphism in intron 2
	PM10	F: 5'-aagcaaggggagccaaga-3' R: 5'-gctgtactgagaaggaaaaattg-3'	60	643	Polymorphism in exon 3
	PM11	F: 5'-tggaaagacagtgggagacatc-3' R: 5'-gcgggtgatggagtaaagtaac-3'	62	764	PCR-RFLP
	PM12	F: 5'-tgcacacgaaaacacaaact-3' R: 5'-tccctatgtcttgtctttttacc-3'	61	654	
	PM13	F: 5'-atggctcctagtcagatttgttt-3' R: 5'-acctcagcagaaacgtcagtg-3'	60	740	
	PM14	F: 5'-caacacaattcaactcaggtataa-3' R: 5'-caagatgttagctgtactgagaa-3'	55	1005	

F and R indicate forward and reverse primer; AT = annealing temperature; RT = reverse transcription; PCR = polymerase chain reaction.

Genomic structure and variation analysis of the pig *RFRP* gene

The obtained cDNA in this study was used for BLAST search (http://mgc.ucsc.edu/cgi-bin/hgBlat) to reveal the genomic organization of pig *RFRP* gene. According to the predicted genome organization, primers P5 to p10 were used to amplify the genomic sequence to identify potential polymorphisms by using 25 DNA templates, and then directly sequenced by BGI-Shenzhen (Guangdong, China). The sequence blast was performed using Seqman program of DNASTAR software package (www.dnastar.com/), and only those variations that presented at least twice were regarded as SNPs.

SNP genotyping by PCR-RFLP

From the obtained SNPs, appropriate enzymes were selected using mapdraw program of DNASTAR software to genotype by PCR-RFLP; the following enzymes were used: *Pst*I, *Sty*I, *Sma*I, *Bsr*I, *Hha*I, and *Hin*fI for SNP of g.45859333T>C, g.45859759C>T, g.45859814G>T, g.45859938A>G, g.45861084T>G, and g.45862152T>A, respectively (P7 and P11-P14). The digestion mixture contained 8 μL PCR products, 1 μL 10X digestion buffer, and 3.0 U enzyme. Digestion was performed at 37°C overnight. The digested PCR products

were electrophoresed on 2.5% agarose gel and visualized using a TFM-40 UV Transilluminator.

Real-Time PCR analysis of RFRP mRNA expression in pig tissues

The 20-μL qPCR mixture contained 1 μL cDNA template, 0.2 μL 10 μm primers, and 10 μL EvaGreen suppermix. qPCR was performed at 95°C for 3 min, followed by 40 cycles of 30 s at 95°C, 30 s at 61°C, and 40 s at 72°C by using BIO-RAD CFX96 (Bio-Rad, USA). A specific PCR band was obtained by performing melting curve analyses. In addition, qPCR products were verified by sequencing on BGI-Shenzhen (Guangdong, China).

The experiments were repeated in triplicate. Quantitative values were obtained from the threshold PCR cycle number (Ct), at which the increase in signal was associated with an exponential growth for PCR products to be detected. The relative mRNA levels in each sample were normalized by β-actin. The relative expression levels of pig *RFRP* gene was indicated by $2^{-\Delta\Delta Ct}$, for which $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{female\ cerebrum}$ ($\Delta Ct = Ct_{target\ gene} - Ct_{ß\text{-}actin}$).

Bioinformatic analysis

The protein and CDS data of RFRP in various species were downloaded from NCBI database according to the BLAST result of Human RFRP. They were human (protein ID of NP_071433, CDS ID of AB040290.1), chimpanzee (XP_001160762.1; XM_001160762.1), marmoset (XP_002751507.1; XM_002751461.1), Rhesus monkey (BAE17051.1; AB193141.1), cattle (NP_776593.1; NM_174168.1), sheep (NP_001120740.1; NM_001127268.1), house mouse (AAK94202.2; AB040289.1), Norway rat (AAK94203.2; AF330059.2), and chicken (AAR14159.1, AY442186.1). Alignment of RFRP was performed using the MEGA 4.1 software (www.megasoftware.net/), and identity percentage was deduced using the DNASTAR software (www.dnastar.com/). Furthermore, a series of online services were used to predict physical properties (http://www.expasy.org/tools/protparam.html), signal peptide (http://www.cbs.dtu.dk/services/SignalP/), and transmembrane domain (http://www.cbs.dtu.dk/services/TMHMM/).

Marker trait association analyses

Association analyses of marker traits were performed using the SAS 8.0 GLM procedure (Liu et al., 2011; Zhang et al., 2012), and the genetic effects were analyzed using the following mixed model:

$$Y = \mu + G + P_i + S_j + e$$

where Y is an observation on the trait, μ is the overall population mean, G is the fixed effect of genotype, P_i is the fixed effect of parity (i = 1, 2, 3, 4, 5, and 6), S_j is fixed effect of breed (j = 1 for Yorkshire and 2 for Landrace), and e is the residual random error. Multiple comparisons were analyzed with least squares means. The values were considered to be significant at $P \leq 0.05$ and presented as least squares means ± SE.

Codon usage bias analysis

The codon usage bias was analyzed using the online service (http://www.bioinformat-

ics.org/sms2), and relative synonymous codon usage (RSCU) was calculated using the following mixed model (Comeron and Aguadé, 1998; Carbone et al., 2003):

$$R_{ij} = k\ (N_j / N_{aa})$$

where R_{ij} refers to the number of times codon j was used for the CDS sequence; i, *k* refer to the quantity of synonymous codons; N_j is the amount of codon *j* in the CDS sequence, and N_{aa} is the total frequency of synonymous codons in the CDS sequence.

RESULTS

The pig RFRP cDNA sequence and online prediction of protein function

The obtained pig RFRP cDNA was 645 bp in length containing 564 bp ORF, 37 bp 5'UTR and 44 bp 3'UTR and it encoded a protein of 188 amino acids (AA; NCBI accession No. JN857935; Figure 1). The cDNA sequence of pig *RFRP* gene showed identities of 81, 68.8, and 76.1% with those of human, mouse, and rat, respectively. Their corresponding protein homology was 69.1, 54.7, and 56.1%, respectively. Prediction indicated that RFRP peptide was an instable and hydrophobic protein, of which the isoelectric point (IP) was 10.34. Moreover, the pig RFRP contained a signal peptide located at LLT-SN and a transmembrane domain located at MEIISSKRFVLWTLAASSLLTSNIFCTD.

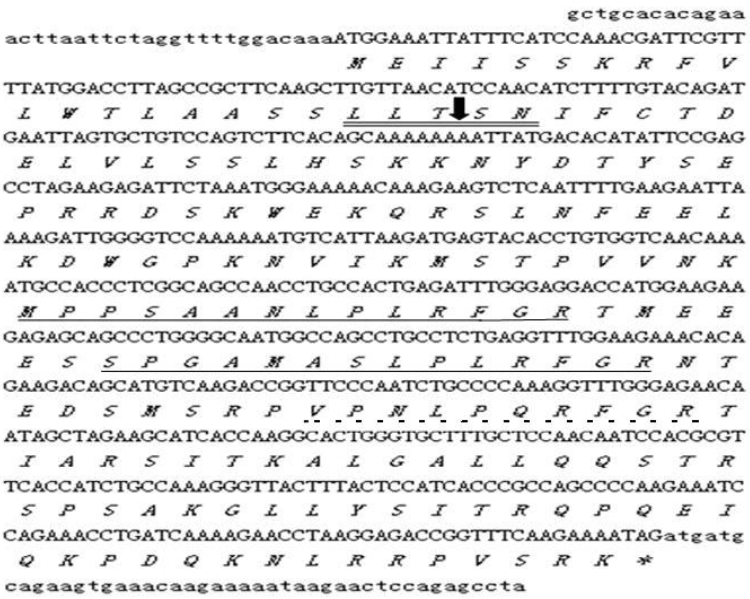

Figure 1. cDNA and deduced amino acid sequences of pig RFRP gene. Capital and italic letters showed amino acids for each codon upside, while asterisk refers to stop codon. Double uderline showed the predicted cleavage sequence of signal peptide, and inverted arrowhead showed cleavage site. The single underline referred to peptide sequence of RFRP-1, which has LPLRF sequence. The broken line referred to peptide sequence of RFRP-3 with sequence of LPQRF. No matter RFRP-1 or RFRP-3, both amino acids ended with GR (Glycine, Arginine).

Homology of RFRP in 10 species

Blast search showed that pig RFRP exhibited higher homology with cattle (84%) and sheep (94.3%; **Figure S1**). The CDS homology was higher than the protein homology among all analyzed species. Moreover, the "RFGR" and similar "RFGR" motifs of "RFamide" structure could be found in the 10 species (**Figure S1**). A total of 31 codons showed bias of usage on the basis of relative synonymous codon usage (RSAU) >1.

Genomic characterization of pig *RFRP* gene

The pig *RFRP* gene is located at 45857868-45862257 nt of pig chromosome 18 (reverse strand) spanning 4517 bp, and it contains 3 exons and 2 introns (Figure 2). The nucleotide sizes of exon 1-3 are 175, 404, and 196 bp, respectively, and they are 1585 and 2157 bp for introns 1 and 2 each. The boundary nucleotides of the 2 introns followed the putative "GT-AG" rule.

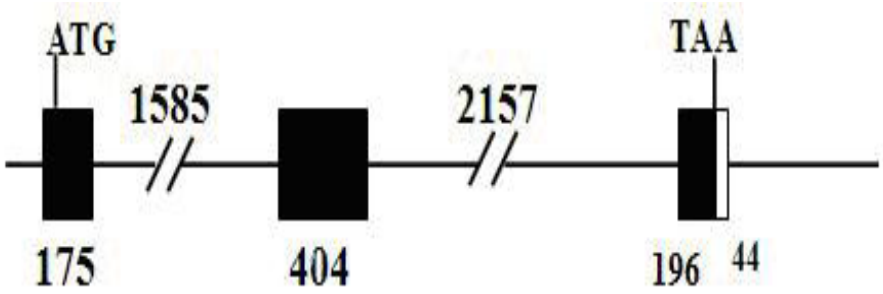

Figure 2. Genomic organization of pig RFRP gene. ATG is initiation codon, and TAA is stop codon. The black boxes indicated exons with nucleotide size showed below. Double bars indicated introns with nucleotide size shown upside. The white box indicated the 3'UTR obtained in this study.

RFRP mRNA expression in various tissues

qPCR showed that pig *RFRP* gene was expressed in most tissues in both females and males (Figure 3; **Table S1**). In females, the highest mRNA was found in the large intestine (2.30 ± 0.275), and then in the uterus (1.36 ± 0.246), liver (1.14 ± 0.224), lymph (1.09 ± 0.0791), stomach (1.05 ± 0.168), cerebrum (1.00 ± 0.0898), subcutaneous fat (8.56×10^{-1} ± 0.212), cerebellum (8.30×10^{-1} ± 0.271), lung (7.60×10^{-1} ± 0.0502), kidney (6.73×10^{-1} ± 0.250), ovary (5.06×10^{-1} ± 0.0906), small intestine (3.87×10^{-1} ± 0.239), abdominal fat (3.77×10^{-1} ± 0.222), oviduct (3.31×10^{-1} ± 0.312), hypothalamus (2.62×10^{-1} ± 0.293), heart (2.44×10^{-1} ± 0.515), back muscle (1.82×10^{-1} ± 0.0332), pituitary (1.39×10^{-1} ± 0.276), back leg muscle (1.34×10^{-1} ± 0.245). The lowest mRNA was found in the spleen (7.60×10^{-2} ± 0.541) and foreleg muscle (2.84×10^{-2} ± 0.674). In males, the highest can be detected in the digestive system of stomach, and then it was found in the liver, cerebrum, hypothalamus, and cerebellum. Interestingly, the reproductive system of testis also had higher mRNA levels than in other tissues.

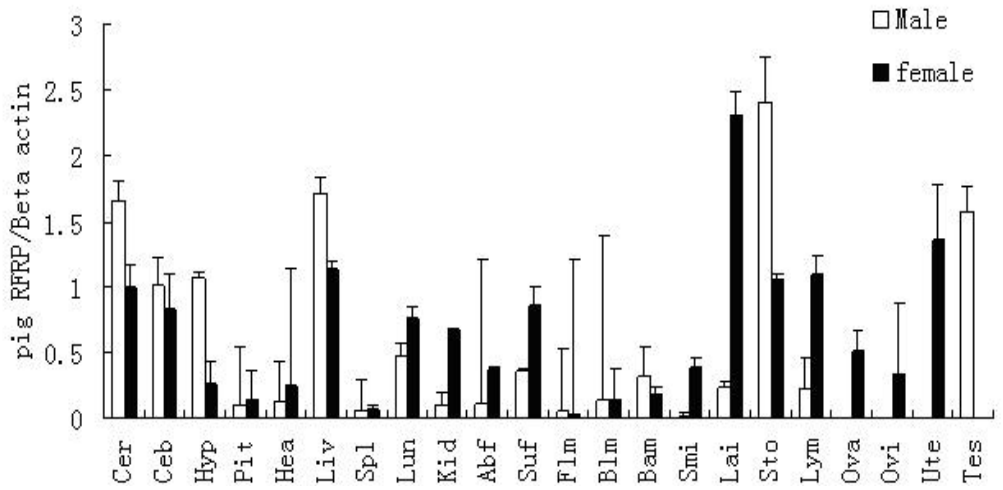

Figure 3. mRNA levels of pig *RFRP* gene in different tissues of male and female Yorkshire. Cer = cerebrum; Ceb = cerebellum; Hyp = hypothalamus; Pit = pituitary; Hea = heart; Liv = liver; Spl = spleen; Lun = lung; Kid = kidney; Abf = abdominal fat; Suf = subcutaneous fat of back; Flm = forth leg muscle; Blm = back leg muscle; Bam = Back muscle; Smi = small intestine; Lai = large intestine; Sto = stomach; Lym = lymph; Ova = ovary; Ovi = oviduct; Ute = uterus, and Tes = testis.

SNPs of pig *RFRP* gene

A total of 25 SNPs and 2 indels were identified in the 4157 bp region of pig *RFRP* gene (Table 2). The mean SNP density was calculated every 154 bp per SNP. Among 9 SNPs in exons, 6 are missense mutations, i.e., g.45859759C>T, g.45859795C>T/A, g.45859796A>G, g.45859822C>T, g.45859938A>G, and g.45862152T>A. Three others (g.45859754G>A, g.45859814G>T, and g.45859922C>T) are synonymous mutations. Moreover, the SNP of g.45859795C>T/A was a multiple allele variation. Since 20 of these SNPs were located at the sites of specific restriction enzymes, they could be genotyped by PCR-RFLP.

Table 2. Single nucleotide polymorphisms (SNPs) of pig RFRP gene identified in this study.

No.	Site	Region	SNP	No.	Site	Region	SNP
1	45859333	Intron 1	T>C	15	45861084	Intron 2	T>G
2	45859349	Intron 1	C>T	16	45861412	Intron 2	C>T
3	45859516	Intron 1	T>C	17	45861489	Intron 2	T>C
4	45859573-45859577	Intron 1	CTTAA>Del	18	45861588	Intron 2	T>C
5	45859608	Intron 1	T>C	19	45861656-45861657	Intron 2	Ins>TT
6	45859626	Intron 1	C>T	20	45861717	Intron 2	T>C
7	45859754	Exon 2	G>A	21	45861823	Intron 2	A>G
8	45859759	Exon 2	C>T	22	45861850	Intron 2	T>A
9	45859795	Exon 2	C>T/A	23	45861893	Intron 2	T>G
10	45859796	Exon 2	A>G	24	45862061	Intron 2	C>T
11	45859814	Exon 2	G>T	25	45862120	Intron 2	C>G
12	45859822	Exon 2	C>T	26	45862127	Intron 2	T>C
13	45859922	Exon 2	C>T	27	45862152	Exon 3	T>A
14	45859938	Exon 2	A>G				

The site of each SNP was determined based on pig genome at chromosome 18, according to November 2009 (SGSC Sscrofa9.2/susScr2) pig BLAT search (http://genome.ucsc.edu/cgi-bin/hgBlat).

Association of the pig *RFRP* gene polymorphisms with reproductive traits

In this study, 6 SNPs were chosen to analyze their associations with reproductive traits. Results showed that only g.45859759C>T was significantly associated with the TBA of second parity ($P < 0.05$; Table 3).

Table 3. Association of 45859759C>T with pig reproduction traits

SNPs	RFLP enzyme	Traits	P value	Least squares mean (LSM) ± standard error (SE)		
45859759C>T	*Sty*I (*Eco*130I)	TBA2	0.0403	11.74 ± 0.45(CC/35)^{ab}	11.40 ± 0.18(TC/237)^{a}	10.55 ± 0.17(TT/238)^{b}

TBA2 referred to the total number of second. The bracketed letters and numbers indicate genotype and individuals. Values within a row with no common superscript letters differ significantly ($P < 0.05$).

DISCUSSION

In this study, the complete CDS of pig *RFRP* gene was isolated by RT-PCR and were submitted to the NCBI database with their protein sequence, and its predicted molecular weight was 21.22 kDa. The encoded pig *RFRP* gene shared identities of 81, 68.8, and 76.1% with its counterparts in human, mouse, and rat, respectively. Nevertheless, interestingly, the homology of pig RFRP protein was lower than that of CDS, and the result was consistent with those found in other species. By using the MSM software, we analyzed a total of 31 codons, of which codon bias was noted in 10 species, and the higher frequency codons were AAA (107), GAA (102), AGA (84), CTG (57), and AAT (56). Like humans and mice, the pig *RFRP* gene also contained 3 exons and 2 introns, and all introns followed the rule of GT-AG. In addition, the pig RFRP peptide also contained 2 forms of RFRP-1 (MPPSAANLPLRF-NH_2) and RFRP-3 (VPNLPQRF-NH_2), just as those in humans (Ubuka et al., 2009).

The expression of *RFRP* gene in various species was very different. In mouse, the *RFRP* gene can be detected in the hypothalamus, pituitary, eyes, brain, testis (Hinuma et al., 2000), and ovary (Singh et al., 2011). In humans and hamster, the tissues of hypothalamus, and pituitary showed a litter higher mRNA level. The high expression of pig *RFRP* gene in reproductive and other tissues indicated its functions in these systems. The pig RFRP gene was found to abundantly expressed in the nervous system (cerebellum, cerebrum, hypothalamus, and pituitary), digestion system (large intestine, stomach), reproduction system (ovary, oviduct, uterus, and testis), and other tissues (liver, lymph, lung, and kidney). In female pigs, the highest expression was found in the large intestine, and then in the uterus. In male pigs, the highest expression was found in the stomach, which also is a digestive organ, and then was in the liver. The lowest mRNA level was detected in the small intestine, while in female pig was forth leg muscle. Regardless of whether male or female pigs were used, both reproductive systems showed a little higher mRNA expression. Accordingly, whether this was related to the feeding actions and reproduction remained unclear in mammals, whereas, in the study of Galvin et al. (1993), the larger uterine capacity was found to be useful for the maintenance of a higher number fetuses.

Until recently, the polymorphisms of pig *RFRP* gene were unclear, except for 3 variations reported in human (Schulz et al., 2002). In this study, 25 SNPs and 2 indels were identified, of which one-third were located in exons, and most of them predominantly changed between A↔G and C↔T (Pettersson et al., 2009). Six SNPs (g.45859333T>C, g.45859759C>T,

g.45859814G>T, g.45859938A>G, g.45861084T>G, g.45862152T>A) were selected to analyze the association with some pig reproductive traits in Yorkshire (334) and Landrace (187) populations. Results showed that only g.45859759C>T was significantly associated with TBA2 ($P < 0.05$). A recent study also revealed the effects of RFRP-3 on the female pig reproductive axis *in vitro* (Li et al., 2013). It was therefore suggested that pig *RFRP* gene are a crucial candidate gene for reproduction traits in pigs.

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Supplementary material

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