Porcine *MAP3K5* analysis: molecular cloning, characterization, tissue expression pattern, and copy number variations associated with residual feed intake

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ABSTRACT. Mitogen-activated protein kinase kinase kinase 5 (MAP3K5) is essential for apoptosis, proliferation, differentiation, and immune responses, and is a candidate marker for residual feed intake (RFI) in pig. We cloned the full-length cDNA sequence of porcine *MAP3K5* by rapid-amplification of cDNA ends. The 5451-bp gene contains a 5'-untranslated region (UTR) (718 bp), a coding region (3738 bp), and a 3'-UTR (995 bp), and encodes a peptide of 1245 amino

acids, which shares 97, 99, 97, 93, 91, and 84% sequence identity with cattle, sheep, human, mouse, chicken, and zebrafish MAP3K5, respectively. The deduced MAP3K5 protein sequence contains two conserved domains: a DUF4071 domain and a protein kinase domain. Phylogenetic analysis showed that porcine MAP3K5 forms a separate branch to vicugna and camel MAP3K5. Tissue expression analysis using real-time quantitative polymerase chain reaction (qRT-PCR) revealed that *MAP3K5* was expressed in the heart, liver, spleen, lung, kidney, muscle, fat, pancrea, ileum, and stomach tissues. Copy number variation was detected for porcine *MAP3K5* and validated by qRT-PCR. Furthermore, a significant increase in average copy number was detected in the low RFI group when compared to the high RFI group in a Duroc pig population. These results provide useful information regarding the influence of MAP3K5 on RFI in pigs.

Key words: Copy number variation; *MAP3K5*; Porcine; RACE; mRNA expression: Residual feed intake

INTRODUCTION

Mitogen activated protein kinase kinase kinase 5 gene (MAP3K5), also known as apoptosis signal-regulating kinase 1, is a serine/threonine kinase that belongs to the MAPK superfamily. Once activated, MAP3K5 can trigger the apoptogenic kinase cascade via activation of C-Jun N-terminal kinase and p38-MAP kinase, resulting in apoptosis and delayed gastric emptying (Kanamoto et al., 2000; Tobiume et al., 2001; Yang et al., 2014). MAP3K5 also plays an important role in oxidative stress, cellular proliferation, and differentiation, and in immune responses (Harada et al., 2006; Hayakawa et al., 2006; Choi et al., 2011; Prickett et al., 2014). Residual feed intake (RFI) is closely associated with tissue growth, apoptosis, differentiation, gastric emptying rate, and immune responses (De Hear et al., 1993; Kim et al., 2007; Smith et al., 2011; Dunkelberger et al., 2015). Therefore, owing to its multi-functional characteristics, previous studies have suggested that MAP3K5 controls feed efficiency, including RFI (Do et al., 2014).

However, the full-length porcine *MAP3K5* cDNA is not available in the current GenBank database. Therefore, the aim of this study was to clone the full-length porcine *MAP3K5* cDNA and investigate the mRNA expression profile of this gene in different tissues. In addition, *MAP3K5* copy numbers were determined and their association with RFI was analyzed in a Duroc population.

MATERIAL AND METHODS

Animals and data collection

All animal care followed the Guidelines for the Care of Experimental Animals established by the Council of China. All procedures in this study were performed according to protocols approved by the Swine Genetics and Breeding Department of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (CAAS, Beijing, China).

Pigs of similar age were placed together in pens. Their diet met or exceeded National Research Council (NRC, 1998) requirements. Daily feed intake (DFI) and body weight (BW) data were collected by Electronic Feed Intake Recording Equipment (FIRE, Osborne, KS, USA). DFI and BW data were collected from 90 days of age (on-test) until a BW of about 100 kg was attained (off-test). The 10th-rib back fat (BF) was measured using an Aloka ultrasound machine (Corometrics Medical Systems, Inc., Wallingford, CT, USA). The average daily gain (ADG) was estimated from the slope of a simple-linear regression of daily BW. DFI was defined as the DFI during the test period. The equation used to predict RFI has been previously described (Cai et al., 2008):

$$RFI = DFI - [b_1 \times (onBW - 30) + b_2 \times (offBW - 100) + b_3 \times metamidBW + b_4 \times ADGA + b_5 \times offBFA + e]$$

where, onWT is defined as on-test BW; offWT is defined as off-test BW; metamidWT is defined as metabolic mid-body weight (average weight raised to the power of 0.75); ADGA is defined as ADG adjusted to 90-180 days of age; and offBFA is the off-test BF adjusted to 100 kg BW. The regression coefficients (b) and regression intercept (e) were computed by a multiple-linear regression model.

Tissue preparation and RNA extraction

The heart, liver, spleen, lung, kidney, muscle, fat, pancreas, ileum, and stomach samples were harvested from Duroc pigs weighing 100 kg (N = 3). All samples were collected, snap-frozen in liquid nitrogen, and stored at -80°C until use. These tissue were used to assess *MAP3K5* gene expression. mRNA from liver tissue was used in rapid-amplification of cDNA ends (RACE) polymerase chain reaction (PCR). Total RNA was extracted using the TRIzol method following the manufacturer instructions. An equivalent amount of RNA from three Duroc pigs was pooled for tissue expression analysis. RNA was stored at -80°C until use.

Cloning of the full-length porcine *MAP3K5*

cDNA was cloned using the PrimeScript™ RT reagent kit (TaKaRa, Tokyo, Japan), according to the manufacturer instructions. Primers were designed based on the predicted porcine *MAP3K5* mRNA sequence (GeneBank accession No. XM_005659186.1) and other conserved sequences of known vertebrate species (Table 1). The cDNA was used as a template, and PCR was performed in a 50-μL volume containing 2 μL cDNA, 200 nM forward/reverse primers, 250 μM deoxyribonucleoside triphosphate (dNTPs), 1 U Taq polymerase, and 25 μL 2X GC Buffer I (TaKaRa). The following PCR cycling parameters were used: 94°C for 5 min, followed by 36 cycles of 94°C for 30 s, an appropriate annealing temperature for 50 s and 72°C for 1 min, with a final extension of 72°C for 10 min. The PCR products were cloned into the T-Vector pMD™ 20 (TaKaRa) and sequenced by SinoGenoMax Co., Ltd.

RACE-PCR was performed using a 3'-full RACE core set with PrimeScript™ RTase (TaKaRa) and a SMARTer™ RACE cDNA amplification Kit (TaKaRa) according to the manufacturer instructions. Primers were designed based on the previously identified middle fragment. For 3'-RACE, the first round of PCR was performed with a gene-specific primer, 3M1F, followed by the second and third nested PCRs with another gene-specific primer, 3M2F. For 5'-RACE, the first round of PCR was performed with a gene-specific primer,

5M1R, followed by the second, third, and fourth nested PCRs with the gene-specific primers, 5M2R, 5M3R, and 5M4R. The products were purified using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver. 3.0 (TaKaRa), cloned into the T-Vector pMD™ 20 (TaKaRa), and sequenced by SinoGenoMax Co., Ltd.

Table 1. Primers used for PCR amplification of *MAP3K5*.

Primer purpose	Primers	Primer sequences (5'-3')	Annealing T (°C)	Product size (bp)
Cloning middle fragment	M1F	TGCCGTAGTGGAGATGAGT	60	296
	M1R	CAGATGGGTCCGAGTAGC		
	M2F	CGCATAATAAGGTCTACTG	58	609
	M2R	CTTACCAAGAAGCTCCAC		
	M3F	AGTTCCAGCCAGTATTTC	55	886
	M3R	CAGATACGAAGGCTGATA		
	M4F	CCTGGAGTATGACTATGGA	53.5	673
	M4R	TTCCCACCTTGAACATCG		
	M5F	GATGTTCAAGGTGGGAATG	53.5	585
	M5R	GAGGCTTGTGACGAGGGT		
	M6F	TCCAGACACGGAGTTGAA	53.5	990
	M6R	GTCTTCATCGGCTCCATT		
	M7F	CTGAGTTCTACTGTATCCCACG	58	786
	M7R	GTCCTCTTCGTTTCGTGT		
	M8F	AAAATGGAGCCGATGAAG	55	800
	M8R	ATTCGGGCAGGTACAACT		
5'-RACE	5M1R	GGTCTCTACTTTCAGTGTCCATG	55	1307
	5M2R	TTCGACCAACAAGGCAATACAT	55	
	5M3R	GCTCGCTTCGTTGATGACATAT	55	732
	5M4R	CGAAGGGTGGCACGGAGAAAGT	55	
3'-RACE	3M1F	TGTGTTTGTAATATGCCCTGAT	55	150
	3M2F	AGTTTCCGGCTTAGAGTACATT	55	

Bioinformatic analysis of porcine *MAP3K5*

NCBI BLAST was used to search for sequence homology and for comparative analyses of the MAP3K5 gene and protein sequence (http://www.ncbi.nlm.nih.gov/BLAST/). The *MAP3K5* open reading frame (ORF) was predicted by ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid composition was estimated using the ExPaSy software (http://www.expasy.org), and the secondary and tertiary structures of the deduced amino acid sequences were visualized using PSIPRED v. 3.3 (http://bioinf.cs. ucl.ac.uk/psipred/) and SWISS-MODEL (http://swissmodel.expasy. org/). InterProScan sequence search (http://www.ebi.ac.uk/interpro/search/sequence-search) was used to predict conserved domains. The TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHM M-2.0/) was used to identify transmembrane domains, and the signal peptides were predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The hydrophilic-hydrophobic properties of the putative porcine MAP3K5 protein were predicted using the ExPaSy ProScale software (http://web.expasy.org/protscale/). The putative amino acid sequence of porcine MAP3K5 was aligned with that of other mammalian species using the CLUSTAL-X program (http://www.Ebi.ac.uk/Tools/msa/clustalw2). A phylogenetic tree was constructed by maximum likelihood analysis using the MEGA 5.2 software (http://www.Megasoftware.net/).

Analysis of tissue expression by qRT-PCR

cDNA was produced by reverse transcription as described above. *MAP3K5* mRNA

expression was measured in different tissues by qRT-PCR. This study was based on the porcine *MAP3K5* mRNA sequence (GeneBank accession No. KT692954). mRNA expression levels were determined using SYBR Green qRT-PCR with an ABI7500 instrument (Applied Biosystems, Inc., Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene to normalize the expression in the different tissues. The primers used in this assay are listed in Table 2. PCR was performed using 1 μL mixed cDNA with the following procedure: 7.5 μL SYBR Select Master Mix (ABI, USA), 5.5 μL RNase-free H_2O, and 0.5 μL forward and reverse primers. Each qRT-PCR cycle was conducted as follows: 50°C for 2 min, 95°C for 5 min, 95°C for 10 s, 60°C for 1 min for 36 cycles. Each independent experiment was conducted in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression (Livak and Schmittgen, 2001).

Copy number variation detection and association with RFI

MAP3K5 copy number variation (CNV) was determined and validated by SYBR Green qRT-PCR using an ABI7500 instrument (Applied Biosystems). Among 526 Duroc pigs, those with either a low (N = 20) or high RFI (N = 20) were selected and ear tissue was collected. Genomic DNA was extracted using the standard phenol-chloroform extraction procedure. DNA was stored at -20°C until use. DNA expression was normalized using the glucagon gene (*GCG*) as a reference. The primers used for the assay are listed in Table 2 and the reaction was performed as described above. Copy number was calculated using the $2^{-\Delta\Delta Ct}$ method (Graubert et al., 2007; Wang et al., 2014), where ΔCt is the cycle threshold of the target region minus that of the control region. Statistically significant differences were calculated by the *t*-test in SAS (2008) software package version 9.2 (SAS Institute Inc., Cary, NC, USA).

Table 2. Primers used for qPCR amplification of *MAP3K5*.

Primer purpose	Primers	Primer sequences (5'-3')	Product size (bp)
mRNA expression	M1F PCR	AGTTCCAGCCAGTATTTC	155
	M1R PCR	CACATGGTGATGGGAGGC	
	GAPDH F	AGGGCATCCTGGGCTACACT	166
	GAPDH R	TCCACCACCCTGTTGCTGTAG	
CNV validation	M1F DPCR	CGCCGTCAGGAACAGATA	91
	M1R DPCR	GCTTGACGATCCAAACCC	
	GCG F	AAGCTTCAAACAGGGGTACAAT	84
	GCG R	CCACTTGGAATGTTACCCTAATG	

CNV = copy number variation.

RESULTS

cDNA sequence and primary structure of porcine *MAP3K5*

A 4385-bp middle fragment was identified by RT-PCR using primers based on the predicted porcine *MAP3K5* mRNA sequence. A BLAST of this fragment revealed high homology with the corresponding sequences from humans, cattle, and mice. The 5451-bp sequence (GenBank accession No. KT692954) of the complete *MAP3K5* cDNA was obtained using the 5'- and 3'-RACE technique, starting with the middle fragment. The full-length cDNA of porcine *MAP3K5* included a 5'-UTR (718 bp), a coding region (3738 bp), and a 3'-UTR (995 bp). The 3738-bp ORF, which encodes a 1245-amino acid protein, was deduced using the

ORF finder software. The splice donor and acceptor consensus sequences were identified at the exon and intron boundaries using the GT-AG rule. In total, 31 exons were identified using the Ensemble BLAST program. A schematic representation of the porcine *MAP3K5* mRNA sequence is depicted in Figure 1.

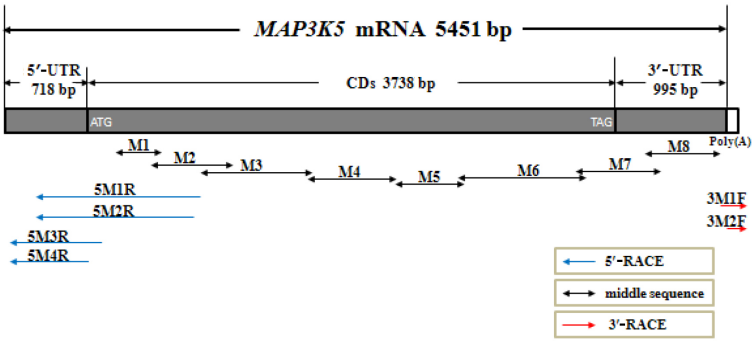

Figure 1. Schematic representation of porcine *MAP3K5* mRNA.

Domain analysis of the deduced MAP3K5 amino acid sequence

The primary structure of the predicted porcine MAP3K5 protein was analyzed and the results revealed that the molecular weight and theoretical isoelectric point were 139.57 kDa and 5.49, respectively. The amino acid composition of this protein is shown in Table 3. Of the different amino acids, the leucine content of the predicted protein was the highest and the tryptophan content was the lowest. The secondary structure of the deduced amino acid sequence is shown in Figure 2. The alpha helix, extended strand, beta turn, and random coil accounted for 53.57, 30.36, 8.03, and 8.03% of the predicted secondary structure composition of the protein, respectively. The three similar types of tertiary structures (662-932, 662-931, 663-932) of the deduced MAP3K5 amino acid sequence were constructed by SWISS-MODEL (Figure 3). Two conserved domains were predicted using the InterProScan sequence search program. The first domain is an unknown function domain DUF4071 (157-537), and the second is a protein kinase domain (677-930) (Figure 4). No transmembrane segments or signal peptide were predicted using the TMHMM Server v. 2.0 and the SignalP 4.1 server software, respectively. Analysis of the hydrophilic-hydrophobic properties showed the same trend as predicted for the transmembrane and signal peptide (Figure 5).

Table 3. Amino acid composition of the porcine MAP3K5 protein.

Name	Count	Count percent	Name	Count	Count percent
Ala (A)	83	6.667	Leu (L)	121	9.719
Arg (R)	58	4.659	Lys (K)	81	6.506
Asn (N)	46	3.695	Met (M)	23	1.847
Asp (D)	74	5.944	Phe (F)	61	4.900
Cys (C)	23	1.847	Pro (P)	56	4.498
Gln (Q)	44	3.534	Ser (S)	105	8.434
Glu (E)	99	7.952	Thr (T)	66	5.301
Gly (G)	74	5.944	Trp (W)	12	0.964
His (H)	31	2.490	Tyr (Y)	37	2.972
Ile (I)	71	5.703	Val (V)	80	6.426

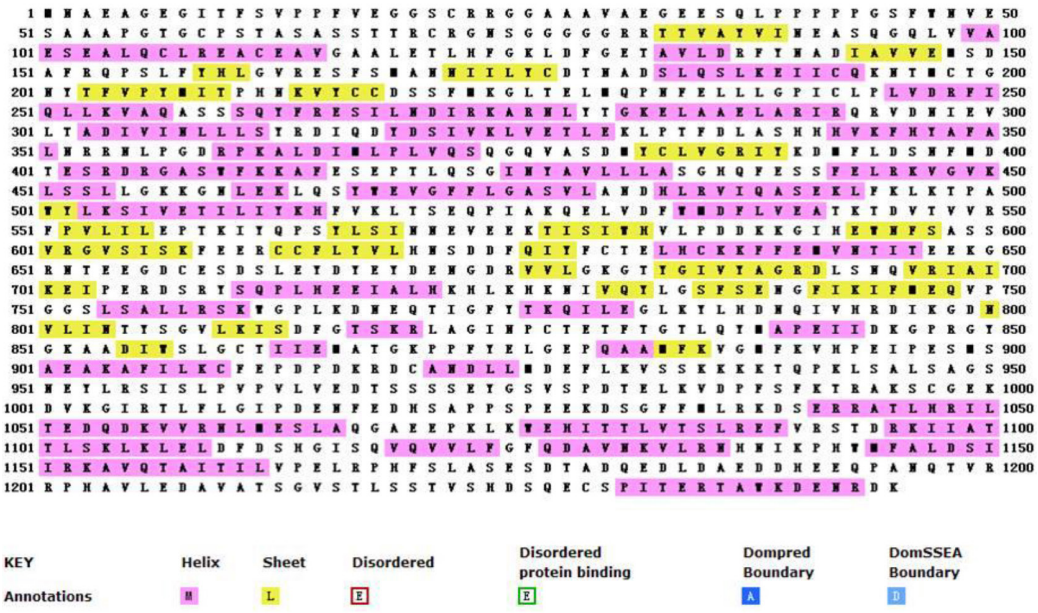

Figure 2. Predicted secondary structure of the deduced amino acid sequence.

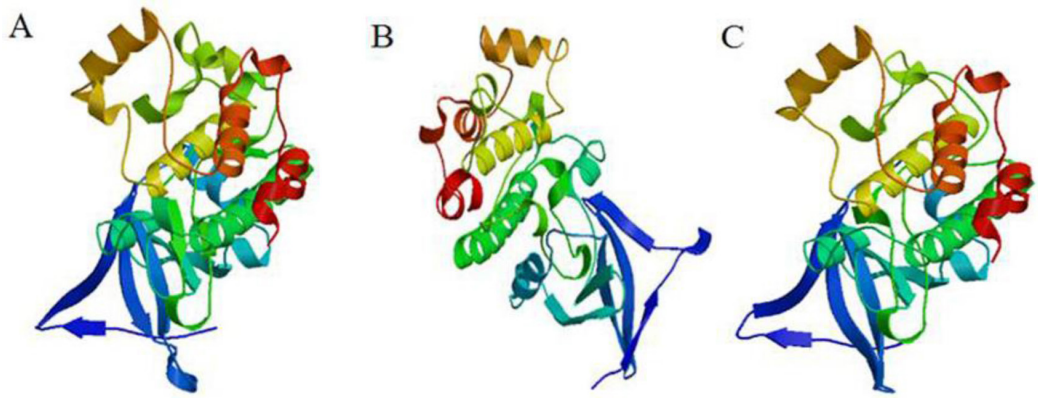

Figure 3. Tertiary structure of the deduced amino acid sequence of MAP3K5 as predicted by SWISS-MODEL. **A.** 662-932; **B.** 662-931; **C.** 663-932.

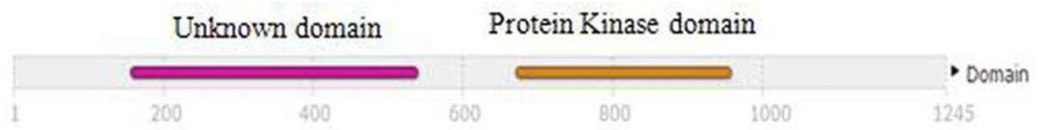

Figure 4. Conserved domain of the deduced amino acid sequence of MAP3K5 as predicted by an InterProScan sequence search. Unknown domain: 157-537; protein kinase domain: 677-930.

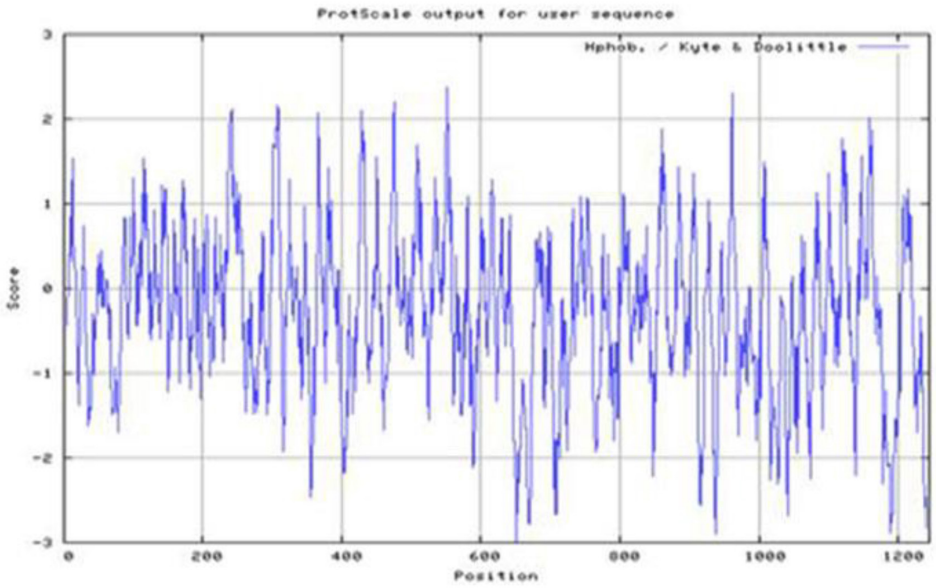

Figure 5. Hydrophilic-hydrophobic properties of the putative porcine MAP3K5 protein.

Homology and phylogenetic analysis

Sequence comparisons revealed homology between the amino acid sequence of porcine MAP3K5 and those from 17 other species. The highest similarity was observed between the MAP3K5 sequence from *Ovis aries* and that from *Camelus ferus* (99%), while the lowest similarity was observed with that of *Danio rerio* (Zebrafish, 84%; Table 4). Based on the alignment results, a phylogenetic tree was constructed using the MEGA 5.2 software, and is shown in Figure 6. Porcine MAP3K5 showed a closer genetic relationship with its *Vicugna pacos* counterpart and was less closely related to its *Gallus* and *Danio rerio* counterparts.

Table 4. Identity matrix of amino acid pairwise comparisons with the porcine MAP3K5 gene.

Species	Accession No.	Identities (%)
Bos taurus	NP_001137553.1	1196/1227 (97)
Bos mutus	ELR53996.1	1149/1173 (98)
Ovis aries	XP_004011568.2	1065/1080 (99)
Orcinus orca	XP_004263831.1	1192/1227 (97)
Vicugna pacos	XP_006200147.1	1203/1227 (98)
Camelus ferus	XP_006188547.1	1068/1080 (99)
Felis catus	XP_011280978.1	1155/1206 (96)
Canis lupus familiaris	XP_533420.4	1186/1227 (97)
Ailuropoda melanoleuca	XP_011217840.1	1192/1227 (97)
Microcebus murinus	XP_012594888.1	1205/1235 (97)
Pan paniscus	XP_003827740.2	1184/1235 (96)
Homo sapiens	NP_005914.1	1202/1235 (97)
Orytolagus cuniculus	XP_008261789.1	1165/1236 (94)
Rattus norvegicus	NP_001264623.1	1155/1242 (93)
Mus musculus	AAI16629.1	1155/1242 (93)
Gallus gallus	XP_419725.3	984/1087 (91)
Danio rerio	NP_001155222.1	1055/1242 (84)

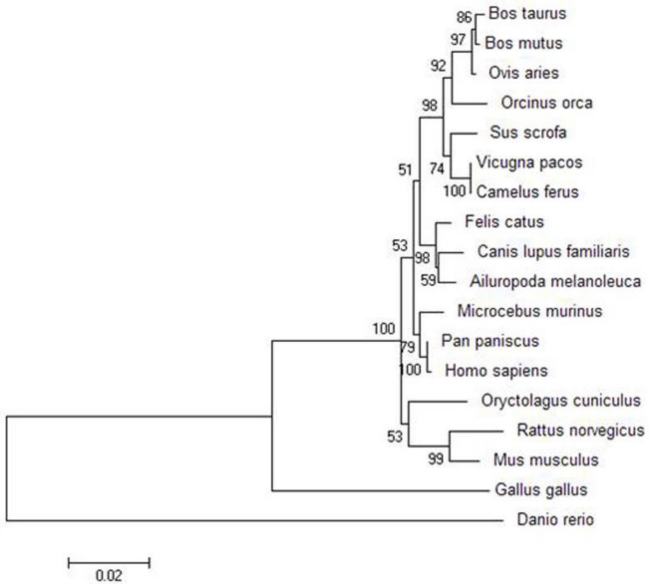

Figure 6. Phylogenetic tree constructed using the MEGA 5 software.

Tissue expression profile of *MAP3K5* in Duroc pigs

Expression of *MAP3K5* mRNA was detected in the heart, liver, spleen, lung, kidney, muscle, fat, pancreas, ileum, and stomach tissues of Duroc pigs (Figure 7). *GAPDH* was used to normalize gene expression. *MAP3K5* mRNA was found to be expressed in all tissues studied from Duroc pig, with the highest level being detected in the heart. In addition, the *MAP3K5* gene was weakly expressed in the lung and pancreas. *MAP3K5* was highly expressed in the back fat, ileum, kidney, spleen, and liver tissues.

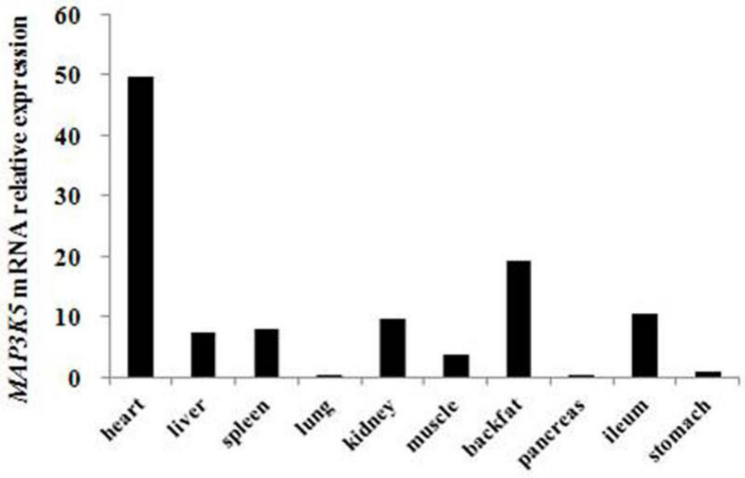

Figure 7. Tissue expression of *MAP3K5* in Duroc pigs.

Relative *MAP3K5* CNV in pigs presenting high and low RFI

Duroc pigs presenting a high and low RFI (N = 20, each) were selected from 526 pigs and ear tissue samples were used to determine the relative copy numbers of *MAP3K5* as described. The RFI data are shown in Table 5. The high and low RFI groups presented significantly different RFIs ($P < 0.0001$). The $2^{-\Delta\Delta Ct}$ method was used to determine relative copy numbers and *GCG* was used to normalize gene expression. A higher relative copy number of *MAP3K5* was detected in low RFI Duroc pigs than in high RFI Duroc pigs. The difference was significant at $P < 0.0001$ (Table 6 and Figure 8).

Table 5. RFI data of the two groups of Durocs.

Breeds	Numbers	RFI (g)	P value
Higher RFI	20	0.280 ± 0.145	<0.0001
Lower RFI	20	-0.213 ± 0.126	

RFI: residual feed intake. Data are reported as means ± SD.

Table 6. Relative copy number of *MAP3K5* in higher and lower RFI Durocs.

Breeds	Number	Relative expression ($2^{-\Delta\Delta Ct}$)	P value
Higher RFI	20	1.012 ± 0.1529	<0.0001
Lower RFI	20	1.575 ± 0.4592	

RFI: residual feed intake. Data are reported as means ± SD.

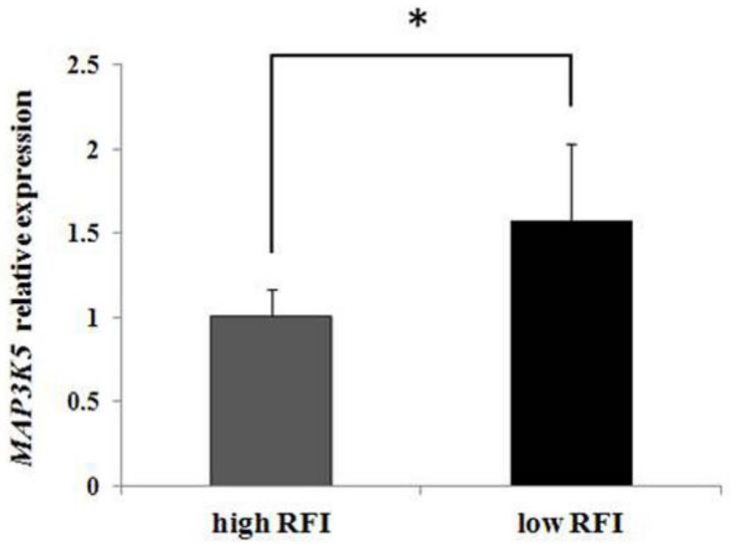

Figure 8. Relative expression of *MAP3K5* in high (N = 20) and low residual feed intake (RFI) Durocs (N = 20) to validate copy number variation (*$P < 0.0001$).

DISCUSSION

The full-length mRNA sequence of the porcine *MAP3K5* gene was not available in GenBank. Therefore, in the present study, we cloned and analyzed the complete porcine *MAP3K5* mRNA sequence from pig liver. The deduced MAP3K5 amino acid sequence was found to contain two conserved domains; an unknown function domain DUF4071 (157-537) and a protein kinase domain (677-930). The unknown function domain DUF4071 was also identified in mouse MAP3K15, MAP3K5, and MAP3K6 (http://www.informatics.jax.org/marker/). The protein kinase domain is an important catalytic domain, which is present in most members of the MAPK family (Scheeff and Bourne, 2005) and involves three predicted tertiary structures. Comparison of protein sequences revealed that porcine MAP3K5 shares more than 97% identity with MAP35K from *Bos taurus*, *B. mutus*, *O. aries*, *Orcinus orca*, *V. pacos*, *C. ferus*, *Canis lupus familiaris*, *Ailuropoda melanoleuca*, *Microcebus murinus*, and *Homo sapiens*. The neighbor-joining phylogenetic tree of MAP3K5 confirmed these findings. Porcine MAP3K5 forms a separate branch to its closest homologs (*V. pacos* and *C. ferus*). These results illustrate that the porcine MAP3K5 protein sequence is most closely related to MAP3K5 from other mammals. Chicken and Zebrafish MAP3K5 were separated by a large evolutionary distance.

MAP3K5 expression levels in Duroc pigs were analyzed by qRT-PCR, and were found to be widely expressed in all tissues examined. The highest *MAP3K5* expression was found in the heart. This is consistent with the reported MAP3K5 expression in mouse embryos (Ferrer-Vaquer et al., 2007). The heart is a muscle that contracts and relaxes at regular intervals. High *MAP3K5* expression in the heart is therefore associated with its high metabolism ability. Higher mRNA levels were detected in back fat, ileum, kidney, spleen, and liver tissues, whereas lower mRNA levels were observed in lung, pancreas, and stomach tissues. These findings are consistent with those of a previous survey indicating that *MAP3K5* is strongly expressed in the condensing nephrogenic mesenchyme of the chicken nephric duct (Ferrer-Vaquer et al., 2007). The spleen is the largest blood filter in the body and combines the innate and adaptive immune systems in a uniquely organized way (Kraal, 1992; Steiniger and Barth, 2000). *MAP3K5* plays pivotal roles in immune responses (Hayakawa et al., 2006).

CNV is considered to be a rich source of genetic diversity in many species (Estivill and Armengol, 2007), and can disrupt gene structure, expression, and function (Henrichsen et al., 2009; Zhang et al., 2009). In this study, we cloned the full-length porcine *MAP3K5* cDNA and determined that the *MAP3K5* gene is located within the *Sus scrofa* chromosome 1 (*SSc1*) region: 30803053-31012340. This region was contained in a reported CNV region, in which different copy numbers were detected between Chinese indigenous and western commercial pig breeds (Wang et al., 2013, 2014; Jiang et al., 2014). . In this study, we found that the relative copy number of *MAP3K5* was significantly different between low and high RFI Duroc pigs. Owing to an insufficient number of tissues being sampled, the association between CNV and mRNA expression was not analyzed in this study. A previous study in cattle reported that the expression of most genes upregulated in low RFI animals was stimulated by the MAPK pathway, which is involved in cellular growth and proliferation (Chen et al., 2011). Serão et al. (2013) noted that the MAPK signaling pathway was over-represented among genes harboring SNPs associated with feed efficiency traits (RFI, FI, ADG, and efficiency of gain) in beef cattle. Hence, similar to what has been reported in previous studies, the present results suggest

that *MAP3K5* might be a good candidate for RFI in pigs.

In summary, we cloned the 5451-bp full-length porcine *MAP3K5* cDNA. *MAP3K5* mRNA expression was the highest in the heart and the lowest in the lung. The relative copy number of *MAP3K5* was higher in low than in high RFI Duroc pigs. This study provides a molecular basis for the further investigation of *MAP3K5* in RFI in pigs.

Conflicts of interest

The authors declare no conflict of interest.

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