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Identification of Molecular Markers for Drip Loss Trait in Thai Commercial Pork

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Drip loss trait causes low quality meat. Thus, the reduction of drip loss in the carcass is one objective in pig breeding programs to meet the consumers' demands for premium-meat. In this work, amplified fragment length polymorphism (AFLP) was used to identifiy molecular markers for drip loss trait of pork. DNA was extracted by using phenol-chloroform method whereas drip loss was measured by using bag method based on gravitational technique. The polymorphism of candidate genes and their association with drip loss were analyzed in Thai commercial pig breeds: purebred Duroc and (Large White × Landrace) × Duroc. Forty eight polymorphic AFLP fragments were observed and showed different frequency distribution between high and low drip loss groups. From these polymorphic fragments, 5 AFLP fragments were successfully reamplified, cloned and sequenced. However, 1 AFLP fragment (*AFLP*1) was characterized and showed homology with porcine WGS nucleotide database. The results revealed that a novel single nucleotide polymorphism (SNP) was found in *AFLP*1 (G>A). Moreover, the nucleotide sequence of *AFLP*1 marker was shown 100% similarity to porcine *UBE3C* gene and SNPs was located at the position of 88,502(G>A) within intron 12 on *UBE3C* gene. These marker was developed into simple co-dominant marker and was genotyped by PCR-RFLP in 1,114 animals. The results indicated that the *AFLP*1 marker were significantly associated with drip loss trait of pork ($p<0.05$). This finding a novel of the importance of porcine *UBE3C* gene (*AFLP*1) as a candidate gene for drip loss trait in Thai commercial pork

Keywords: AFLP, Drip loss, Gene, Pork, *UBE3C*

Introduction

Nowadays, meat quality is in focus worldwide. One of the reasons that causes low quality meat is drip loss trait. It is important in pig production as it

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influences nutrition value, palatability, consumers' perception, weight, technological properties, and especially, the monetary loss. Thus, the reduction of drip loss in the carcass is one objective in pig breeding programs to meet the consumers' demands for high quality meat. The drip loss trait is a complex trait controlling by polygenic and environmental factors. By conventional method, the genetic improvement of drip loss trait is difficult because the heritability is low (Borcher *et al*., 2007; Gjerlaug-Enger *et al*., 2010) and possibly measurable only after slaughter, genetic markers can provide scientific evidence for subsidiary selection to advance the reduction of drip loss in pork. A scan of the pig genome has revealed knowledge on the genetic background of quantitative trait loci for drip loss trait (Jennen *et al*., 2007). Despite this trait has been extensively emphasized, there was no unambiguous causative mutation available.

Objective: To identify molecular markers for drip loss trait in Thai commercial pig breeds.

Materials and methods

Animals

The *Longissimus dorsi* muscle tissues between the 9^{th} and the 10^{th} ribs were cut from a total hot carcasses of 1,114 Thai commercial pig breeds including Duroc (n=419) and (Large White × Landrace) × Duroc (n=695), then store at 4°C for 24 h.

pH and Drip loss measurement

The pH of *Longissimus dorsi* muscle was obtained at 24 h postmortem using an Orion model 720A pH meter fitted with a Ross sure flow 81-72 electrode (Orion Research, Boston, MA). Drip loss trait was measured by using the bag method based on gravitational technique (Honikel, 1998). Meat samples were weighed before (Wd1) and after (Wd2) incubation at 4°C for 24 hours hanging in chambers and drip was expressed as the percentage of total weight lost as follow:

$$\text{Drip loss (\%)} =)Wd1 - Wd2(/ Wd1 \times 100$$

DNA extraction

Genomic DNA was extracted from muscle tissue samples using phenol-chloroform method (Sambrook and Russell, 2001). The concentration of DNA sample was measured with Nanodrop 2000c spectrophotometer (Thermo Scientific, USA) and the integrity of DNA was checked on 1% agarose gel.

Genomic analysis

1) AFLP Analysis

Screening of AFLP to identify *AFLP* markers could be divided into two parts: (I) Screening of AFLP primer combinations from DNA sample pools. (II) The *AFLP* markers were identified in each 20 individual samples of high and low drip loss groups by using selected primers form the results of part I.

During the AFLP analysis, the 20 individuals within the high drip loss group was used to make four high DNA pools by adding equal volume (50 ng) of DNA from each of 5 individuals to a pool. In the same way, the 20 individuals within the low drip loss group was used to make four low DNA pools. These eight DNA pools were performed using adapter and primer sequence according to Ajmone-Marsan *et al*. (1997). In brief, the 250 ng of genomic DNA was digested with two enzymes: FastDigest® *Eco*RI and FastDigest® *Taq*I (Fermentas, USA) based on manufacturer instructions. The digested products were ligated to 50 µmol *Eco*RI-Adapters (F:5'-CTCGTAGACTGCGTACC-3' R:5'-AATTGGTACGCAGTCTAC-3') and 50 µmol *Taq*I-Adapters (F:5'-GACGAT GAGTCCTGAC-3' R: 5'-CGGTCAGGACTCAT-3') in 30 µl of solution containing 6 U T4 ligase and 46 mM ATP under incubation overnight at 4 °C. The ligated FNA templates were adjusted to 10 ng/µl and diluted 1:5 with dH_2O for pre-selective amplification.

The pre-selective amplification PCR condition was: 10 ng of DNA template, 1X *Taq* Buffer (20mM Tris–HCl, pH 8.4, 50mM $(NH_4)_2SO_4$; Fermentas), 3.0mM $MgCl_2$, 0.25mM each of the four dNTPs, 0.25U *Taq* polymaerase (Fermentas), 4 pmol of pre-selective amplification *Eco*RI primer (E-A: 5'-GACTGC GTACCAATT CA-3') and 4 pmol of pre- selective amplification *Taq*I primer (T-C: 5'-GATGAGTCCTGACCGAA-3') with a total volume of 25 µl. The PCR program was as follows: 3 min at 94 °C, 20 cycles of 30s at 94 °C, 1 min at 56 °C and 30 s at 72 °C, followed by 5 min at 72 °C, and ended at 4 °C. PCR products were analyzed by 6% polyacrylamide gel electrophiresis, stained with ethidium bromide and photographed. The

preamplification products were diluted 1:20 with dH_2O and then used as selective-amplification DNA templates.

For selective amplification, eight *Eco*RI primers and eight *Taq*I primers (Table 1) were used, resulting in 64 primer combinations. The following PCR reaction mix was used: 2.5 µl of diluted pre-selective amplification products, 1X *Taq* Buffer (20mM Tris–HCl, pH 8.4, 50mM $(NH_4)_2SO_4$; Fermentas), 3.0mM $MgCl_2$, 0.25mM each of the four dNTPs, 0.25U Taq polymerase (Fermentas) and 2 pmol of selective-amplification *Eco*RI primer and 2 pmol of selective-amplification *Taq*I primer in a total volume of 12.5 µl. A touchdown thermal protocol was used in the selective PCR amplification. The selective-amplification products were denatured and loaded into 6% denature gel electrophoresis then visualized by silver staining.

The differences between high pools and low pools of potential *AFLP* markers were then selected for individual AFLP using the same protocol as described above. The validation was performed on the 20 high drip loss and 20 low drip loss samples and the presence or absence of AFLP bands of interest was scored individually.

Table 1 Primers used for AFLP analysis (Ajmone-Marsan *et al*., 1997)

Primer	Sequence (5´ to 3´)	Primer	Sequence (5´ to 3´)
E-02	GACTGCGTACCAATTCACC	T-03	GATGAGTCCTGACCGACAC
E-03	GACTGCGTACCAATTCACG	T-04	GATGAGTCCTGACCGACAG
E-06	GACTGCGTACCAATTCAGC	T-05	GATGAGTCCTGACCGACAT
E-07	GACTGCGTACCAATTCAGG	T-06	GATGAGTCCTGACCGACCA
E-12	GACTGCGTACCAATTCATC	T-20	GATGAGTCCTGACCGACGA
E-13	GACTGCGTACCAATTCATG	T-22	GATGAGTCCTGACCGACTG
E-15	GACTGCGTACCAATTCAAG	T-23	GATGAGTCCTGACCGACGT
E-16	GACTGCGTACCAATTCAAC	T-25	GATGAGTCCTGACCGACTA

2) Identification of DNA sequence

To identify the single nucleotide polymorphism of SSCP fragments, the PCR product of homozygous individuals with different genotypes were ligated into vectors. The ligated PCR products were used for transform to *Escherichia coli* bacteria. The positive clones were checked by M13 PCR and sequenced.

2.1) Ligation

The PCR fragments were ligated with the pGEM®-T vector (Promega, USA). The ligation reaction was performed in 5 µl of volume, containing 2.5 µl of 2× ligation buffers, 0.5 µl of vector, 1.5 µl of PCR product and 0.5 µl of T4 DNA ligase. The reaction was incubated overnight at 4 ºC

2.2) Transformation

For transformation of recombinant DNA, 5 µl of ligated mixture and 50 µl of the DH5α *E. coli* competent cells were mixed and incubated on ice for 15 min. Heat shock was performed in a water bath at 42 ºC for 90 sec, followed by immediately transfer on ice for 2 min. Then 650 µl of LB-broth (without ampicillin) was added and the mixture was shaken at 200 rpm at 37 ºC for 2 hrs. The cell solution was plated on two ampicillin LB-agar plates as selective medium. The medium contained 20 µl of X-Gal solution and 20 µl of IPTG solution and then incubated overnight at 37 ºC. Five white colonies, expected to contain an insert, were picked from each plate along with a blue colony and confirmed by PCR analysis. White-blue colour of β-galactosidase activity was used as indicator for insert and non insert colony screening.

2.3) PCR analysis of the insert fragment

To identify clones containing an insert, one blue- and five white-colonies of each DNA fragment were picked and suspended in 30 µl of 1 x PCR buffer. The bacterial suspensions were boiled at 95 ^{o}C for 15 min and 10 µl of the lysed bacterial solutions were utilized as DNA templates. PCR reactions were performed as standard protocol and M13 primers (forward: 5´-TTGTAAAACGACGGCCAGT-3´, reverse: 5´-CAGGAAACAGCTATGACC-3´) were used to amplify the insert DNA fragment with 59 ^{o}C annealing temperature and 70 ^{o}C for elongation step. Aliquots of 5 µl of PCR product were electrophoresed on a 1 % (w/v) agarose gels (with ethidium bromide) in 1 x TAE buffer. A ladder DNA marker (100 bp) was used as a reference for fragment size. The gel was photographed under UV- transilluminator. The positive clone was cultured overnight at 37 ºC in 5 ml LB-broth ampicillin. The plasmid DNA was extracted by using GenElute™ Plasmid Miniprep Kit (Qiagen) for sequence analysis.

2.4) DNA Sequencing

Sequencing was carried out on a CEQ 8000 Genetic Analysis System (Beckman Coulter) using a Dye Terminator Cycle Sequencing (GenomeLab™ DTCS) Quick Start Kit (Beckman Coulter). The PCR sequencing reaction was as the following: 50 fM of plasmid DNA, 1 µl of forward primer M13 (1.6 pM), 2 µl of DTCS mix and distilled water added to 10 µl. The sequencing PCR was carried out for 30 cycles at 96 ºC for 20 sec, 58 ºC for 30 sec and 60 ºC for 4 min, holding at 4 ºC. Then the sequence reaction was added with 5 µl of stopping solution (2 µl of 3 M, pH 5.2 sodium acetate, 2 µl of 100 mM Na_2-EDTA, pH 8.0 and 1 µl of 20 mg/ml glycogen). The DNA sequence was precipitated with 60 µl of cold ethanol (-20 ºC). The mixture was centrifuged at 14,000 rpm at 4 ºC for 15 min and supernatant was removed. The

PCR pellet was washed with 1 ml of 70 % cold ethanol 2 times and centrifuged at 14,000 rpm for 2 min at 4 ºC. The pellet was dried for 10 min at room temperature and resuspended with 40 µl of sample loading solution (Beckman Coulter, USA). The DNA solution was analyzed on the CEQ 8000 Genetic Analysis System automated sequencer (Beckman Coulter, USA).

3) SNPs genotyping

After uncovering polymorphisms on the candidate genes, molecular genetic technique based on PCR-RFLP was used to establish protocols for large scale genotyping of DNA samples. The suitable primers (Table 2) were designed for amplifying PCR fragments from genomic DNA. The PCR mixtures contained 1 µl of DNA sample, 1 µl of 10× PCR buffer (Fermantas), 1.2 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (2.5 mM), 0.4 µl of each primer (10 pM/µl) and 0.2 µl of Taq polymerase (5 units/µl, Fermantas), distilled water was added to 20 µl. The PCR conditions used were 38 cycles of 94°C for 30 s, 56-62°C for 30 s, and 72°C for 30 min, followed by 5 min at 72°C. Five microliters aliquots of the PCR products were speared on 1% agarose gel electrophoresis, stained with ethidium bromide. The polymorphism on PCR fragments were detected by restriction enzyme (Table 3.3). The digestion reaction contained 2.5 µl of PCR product, 0.5 µl of 10× buffer (Fermantas) and 1 unit of restriction endonuclease. Distilled water was added to 5 µl and covered with mineral oil. Then the mixture was incubated at 37°C overnight. The digested products were speared on 8 % polyacrylamide gel electrophoresis and stained with silver staining.

Table 2 Details of *UBE3C* and *AFLP*1 genes with regard to primers, annealing temperatures, restriction enzymes and references

Genes	**Primers**	**Annealing, ºC (size, bp)**	**Restriction Enzymes**
*AFLP*1	F: TATGCCCACCTTAGAAGGA R: GCTTGCTGCTGCTCTTTTAG	60 (290)	*Hin*6I

Statistical analysis

All statistical tests were performed with program R (Fox *et al*., 2009). Allele and genotype frequencies were calculated. Association analysis of *AFLP* markers and drip loss trait was examined using general linear model (GLM).

The model included breed and sex as the fixed effects and pH as a covariance as below. All statistical tests were considered significant at $p<0.05$.

$$Y_{ijk} = \mu + breed_i + sex_j + AFLP_k + \beta (pH_{ijk} + \overline{X}) + e_{ijk}$$

where:

y_{ijkl}	=	observed values of phenotypes
μ	=	population means of measurements
$breed_i$	=	fixed effects of breeds (i = 1-2)
sex_j	=	fixed effects of sexes (j = 1-2)
$AFLP_k$	=	effects of genotypes of *AFLP* marker (l = 1-3)
pH_{ijk}	=	covariance of pH
$\overline{X}$	=	mean of pH
β	=	regression value adjusting effects of X for Y
e_{ijk}	=	random error effects

Additionally, additive effect (a) was estimated by comparison of the means of the trait value for homozygote as follows: $a = ½(AA – BB)$. The dominant effect (d) for alleles A and B was calculated from the means for three genotypes as follows: $d = AB – ½(AA + BB)$. The estimated effects were tested by t-test on significant deviation from zero.

Results and Discussions

Genotype frequencies of UBE3C (*AFLP*1)

1) Screening for polymorphism on candidate marker

The AFLP analysis of 40 individual DNA samples could reveal 1 candidate marker that was associated with drip loss. This 1 *AFLP* marker was successfully amplified, sequenced, and identified polymorphisms. Variations amongst the high and low drip loss individuals were found in fragments *AFLP*1. Five nucleotide substitutions were found in a fragment of *AFLP*1 (G>A). The marker of *AFLP*1 was g.88,502 G>A. Furthermore, when the nucleotide sequences of *AFLP* marker (*AFLP*1) were screened against the NCBI and Ensembl databases for similarity to known sequences the results revealed that the *AFLP*1 sequence showed similarity (100%) to SS_WGS-1200e16.p1k (GenBank accession number: ti:1420233834(and located on SSC18 at position 734007 – 734296 within intron 12 on *UBE3C* gene.

2) Development of simple DNA marker

After discovering the polymorphism in pig *AFLP*1 marker, a simple molecular technique based on PCR-RFLP was developed for genotype DNA sample as described below. For PCR-RFLP analysis, the DNA fragments that that were shorter than 50 bp could not be taken into consideration because they were near the DNA detection threshold using silver nitrate staining.

2.1) Genotyping of porcine AFLP marker

The polymorphism of *AFLP*1 marker in the 290 bp PCR fragment was genotyped using restriction enzyme *Hind*6I. Two different alleles were found. Allele A has no recognition site for *Hind*6I and shows an undigested PCR product (290 bp). Allele G has recognition site for *Hind*6I and showed fragment of 176 and 114 bp after digestion.

2.2) Genetic variations of AFLP marker

Genetic variations of *AFLP* markers were genotyped in 1,114 Thai commercial pigs. One polymorphic site of one *AFLP*1 gene was found to be segregation in Thai commercial pig breeds. The genotypic frequencies of *AFLP*1 (AA, GA and GG) were 12, 84 and 323 in Duroc and 153, 458 and 84 in crossbred, while allelic frequencies of this marker were 0.13 and 0.87 in Duroc and 0.55 and 0.45 in crossbred for allele A and G, respectively. (Table 3) The novel nucleotide sequences of *AFLP* marker (*AFLP*1) was screened for similarity to known sequences in NCBI and Ensembl databases. The result revealed that *AFLP*1 sequence showed similarity (100%) with *UBE*3*C* gene. It is an enzyme related to the process of ubiquitylation (Rotin and Kumar, 2009).

Table 3 The genotypic and allelic frequencies of *AFLP*1 marker

***AFLP*1 marker**	**Genotypic frequencies**			**Allelic frequencies**	
	AA	**GA**	**GG**	**f(A)**	**f(G)**
Duroc	12	84	323	0.13	0.87
(Large White × Landrace) × Duroc	153	458	84	0.55	0.45

Effect of UBE3C

The *AFLP*1 marker was associated with drip loss in crossbred pigs. However, there was no association in Duroc. The pig with genotype GG and GA had higher drip loss than genotype AA about 1.62 and 1.69%, respectively. It provided additive effect and dominant effect about 0.81±0.28% and 0.88±0.33, respectively. (Table 4) The *AFLP*1 marker or *UBE*3*C* gene was associated with

drip loss in pork. Because ubiquitin plays significantly in mammals (pigs) for a normal glucose and lipid metabolism concerned in the ubiquitin-proteasome pathway. (Nakamichi *et al*., 2009; Lee *et al*., 2010; Pasaje *et al*., 2011) and ubiquitin-proteasome pathway affect water holding capacity and drip loss as well. (Huynh *et al*., 2014)

Table 4 Association of *UBE3C* gene with drip loss trait

Breeds (N=1,114)	Genotypes			Effects	
	TT	CT	CC	additive	dominant
Duroc	-	7.83±0.26[b]	8.75±0.24[a]	-	-
LW×LD×D	-	6.39±0.34[b]	7.83±0.17[a]	-	-

D = Duroc, LW×LR×D = (Large White × Landrace) × Duroc
Least square mean ± standard error values with different letters in the same row are significantly different (a,b $p<0.05$) **$p<0.01$

Conclusion

The nucleotide sequences of *AFLP* marker (*AFLP*1) were screened for similarity to known sequences in NCBI and Ensembl databases. The results revealed that *AFLP*1 sequence showed similarity (100%) with *UBE*3*C* gene. It was associated with drip loss in crossbred pigs.

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