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# *MC1R*, *KIT*, *IGF2*, and *NR6A1* as markers for genetic differentiation in Thai native, wild boars, and Duroc and Chinese Meishan pigs

**P. Klomtong[1], K. Chaweewan[2], Y. Phasuk[1] and M. Duangjinda[1]**

[1]Department of Animal Science, Khon Kaen University, Khon Kaen, Thailand
[2]Department of Livestock Development, Ministry of Agriculture and Cooperatives, Bangkok, Thailand

Corresponding author: M. Duangjinda
E-mail: monchai@kku.ac.th

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**ABSTRACT.** Mutations in melanocortin 1 receptor (*MC1R*) gene and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*) gene have been shown to affect coat color patterns in pigs. Additional functional marker genes, such as insulin like growth factor-2 (*IGF2*) and orphan nuclear receptor, germ cell nuclear factor (*NR6A1*), have been described for variations in factors such as fat deposition, litter size, and vertebra number in pigs. In this study, we investigated 129 pigs representing 4 breeds: Thai indigenous, classified into black (similar to Raad or Ka done pig) and black and white (similar to the Hailum and Kwai pig) coat color types; wild boar; Duroc; and Chinese Meishan. Mutations of *MC1R*, *KIT*, *IGF2*, and *NR6A1* were detected using polymerase chain reaction-restriction fragment length polymorphism. The genotypes variation in *MC1R* and *KIT* genes could be used to differentiate four groups of coat color: solid black, black and white, red, and wild type. For *IGF2,* the GG genotype was present in wild boar only; for *NR6A1* the TT genotype was found only in Duroc pigs. We identified novel 14-bp deletions in *KIT* that were associated with black and white coat color in

 

Thai indigenous pigs. Insights into variations in genes presented in this study will be useful in future developmental breeding programs for the Thai native pig.

**Key words:** Coat color; Meat quality; Vertebra number; PCR-RFLP; Native pig breeds

## INTRODUCTION

Animal breed identification is important for a variety of reasons. Genetic conservation of domestic livestock usually focuses on maintaining pure breed populations, especially within local and native animal breeds (FAO, 2007). Many small-scale farmers tend to raise more crossbred animals within exotic breeds than purebred animals, resulting in the loss of genetic diversity in native animal breeds. For traceability of meat products, breed identity is important to confirm and guarantee meat product origins and create consumer confidence; this is especially true when consumers are asked to pay a premium price for a meat product. Such a market structure incentivizes farmers to plan breeding strategies and implement herd health programs (D'Alessandro et al., 2007).

Thai native pigs are lard type breeds that, compared to crossbreed pigs, have poor growth rates; lower loin eye area and carcass length traits; higher back fat thickness; and produce small litters (Nakai, 2008). However, native pigs adapt well to a tropical climate, maintain a high utilization of low-quality feed, are perhaps more resistant to disease and internal parasites, and thrive under semi-intensive and intensive management systems (Rattanaronchart, 1994). Additionally, consumers report that Thai native pig meat has better texture and taste than exotic pig meat (Vasupen et al., 2004). Thai native pigs are classified into four breeds according to physical appearance and region of origin: Raad, Hainan, Puang, and Kwai (Rattanaronchart, 1994). In an attempt to improve growth and reproductive performance, Thai farmers often crossbreed native pigs with Duroc and Chinese Meishan breeds. Similarly, Thai native pigs of Thailand's Northeastern hill tribes are frequently crossed with wild boars. As a result, the Thai purebred native pig is being lost. For the purpose of conservation, we propose that means of identification, through genetic signature or breed detection, be developed for the Thai native pig.

Means of differentiating between pig breeds include coat color as well as other characteristic traits, such as fat type, meat texture, number of vertebral bodies, and teat number (Kim et al., 2004; Yang et al., 2009; Alves et al., 2012; Fontanesi et al., 2014; Molnár et al., 2014). The application of genetic DNA sequencing methods is one of the most powerful means of identification and traceability of animal breeds. Such methods include microsatellite and mitochondrial markers; coat color loci; and single nucleotide polymorphisms (SNPs) combination of coat color genes (Alves et al., 2009; Fontanesi and Russo, 2013; Oh et al., 2014). Microsatellite markers have high polymorphic values and employ a large number of microsatellite loci that can be used for breed identification. Scoring of a microsatellite allele may be complicated by a high number of alleles per locus, or other variance factors (Fernandez-Silva et al., 2013). Alves et al. (2009) reported nine mitochondrial DNA sequence variations that are required for assuring authenticity of Duroc and Iberian pigs. However, mitochondrial DNA revealed genetic signature signifying maternal origin only, and was unable to distinguish between purebred and crossbred animals. Furthermore, polymerase chain reaction (PCR)-restriction fragment length polymorphism markers can be detected through the presence of different fragment sizes following digestion with a restriction enzyme. A co-dominate marker shows

both alleles in heterozygous genotypes and is highly polymorphic. These markers can therefore distinguish heterozygous from homozygous genotypes in plants and animals, and may further be used to investigate purebred and hybrid animals within different genetic species (Kijas et al., 1998; Koutsogiannouli et al., 2010).

Polymorphism of the coat color gene may effectively differentiate purebred and crossbred animals. The melanocortin 1 receptor (*MC1R*) gene, located on chromosome 6 in pigs, controls melanogenesis and is expressed in melanocytes of hair follicles. Mutations in *MC1R* determine five coat color variations (extension locus, *E*) in pigs: wild type ($E^+$), dominant black ($E^{D1}$ and $E^{D2}$), black spotting ($E^P$), and red (*e*), (Kijas et al., 1998). The v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*) is located on pig chromosome 8, and is involved in migration and development of melanocytes from neural crest-derived precursor cells (Fontanesi and Russo, 2013). Previous studies of mutations of the *KIT* gene have determined variations in coat color pattern in pigs; these are: dominate white color (*KIT* duplication and deletion with a subsequent splice mutation at intron 17; duplication without splice mutation at intron 17) (Pielberg et al., 2002); belt phenotype (2678C>T SNP in exon 19 of *KIT)* (Xu et al., 2006); and black color (g.276G>A and g.295A>CSNP in intron 19 of *KIT*) (Chung and Chung, 2010). Moreover, Koutsogiannouli et al. (2010) and Marklund et al. (1998) reported that wild boar hybrid or large white intercross pedigrees could be discriminated by characterization of *MC1R*, and of the interaction between *MC1R* and *KIT*. Other genes have been found to control character traits in pigs, such as insulin like growth factor-2 (*IGF2*) and orphan nuclear receptors and germ cell nuclear factor (*NR6A1*); these have also been used to identify pig breeds. Insulin like growth factor-2, which is mapped on pig chromosome 2, has a primary effect on muscle growth and fat deposition. A SNP variation in *IGF2,* at intron 2 g.3072 A, was highly frequent in Duroc pigs but absent in the Iberian pig (Vykoukalová et al., 2006; Alves et al., 2012). The gene *NR6A1*, mapped on chromosome 1, determines the number of vertebrae in pigs. With respect to SNPs in *NR6A1*, g.299084751 C>T (p.Pro192Leu) homozygous genotype (TT) has been found to be prevalent in Duroc, large white, and landrace pigs, whereas Chinese indigenous and wild boar breeds demonstrated greater frequencies of the CC genotype (Yang et al., 2009; Fontanesi et al., 2014). While *MC1R* and *KIT*, including *IGF2* and *NR6A1*, have been studied in other native pig breeds, these genes have not been studied in Thai native pigs, and methods for the identification of breeds within the Thai indigenous pig have not been reported. Therefore, the objective of this study was to investigate the genetic variants of *MC1R* and *KIT*, including *IGF2* and *NR6A1* genes, within two Thai native pig breeds: black coat color, and black coat with white at belly and leg phenotypes.

## MATERIAL AND METHODS

### Samples and DNA preparation

Thai indigenous and exotic and wild boar pigs were analyzed in the study (N = 129). Samples were collected from Thai indigenous pig breeds in North and Eastern Thailand, and classified into two types based on coat color: black (or TNB, similar to Raad or Ka done pigs; N = 35); and black and white (or TNBW, meaning saggy belly with long and straight snouts, similar to Hailum and Kwaipigs; N = 20). Samples from wild boar (WB; N = 14) and two exotic breeds Duroc (DU; N = 35) and Chinese Meishan (MS; N = 25) were also collected (Figure 1).

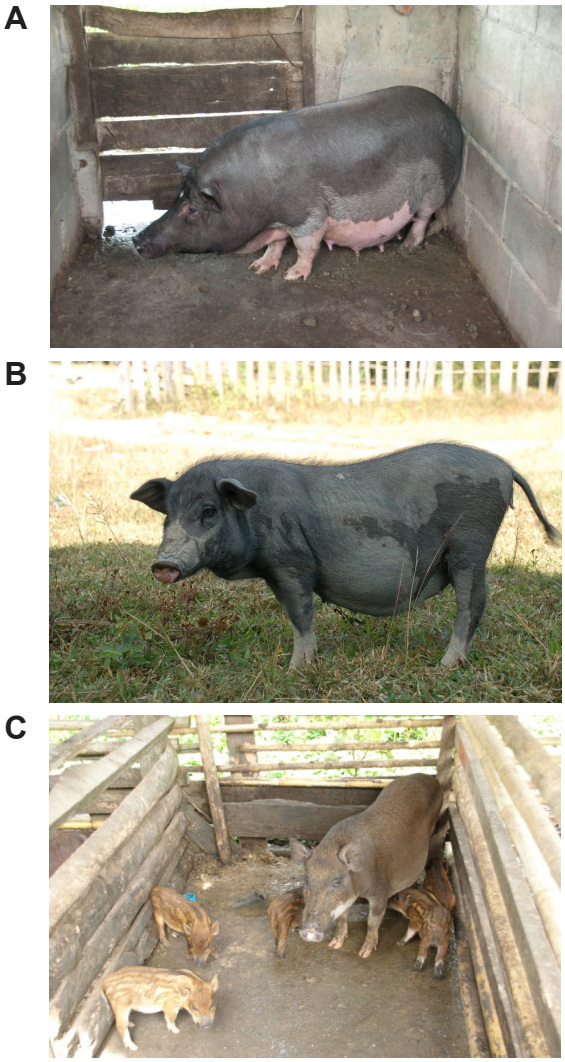


**Figure 1.** Coat color patterns of pig breeds in Thailand. Thai indigenous pigs with black-white (**A**) and black (**B**) coat colors; and wild boar, with bristly coats and a thick underlying brown pelage (**C**), raised near the hill tribes in Eastern Thailand.

Blood samples were collected into 1.5-mL tubes containing an anticoagulant, and genomic DNA was extracted from blood using a modified procedure from Goodwin et al. (2007). The concentration of DNA for each sample was determined using a NanoDrop2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples (50 ng DNA) were stored at -20°C for subsequent use.

## PCR of *MC1R*, *KIT*, *IGF2*, and *NR6A1*

For PCR, solution mixtures (10 µL) contained 1 µL 50 ng genomic DNA; 1 µL 10X PCR buffer; 1 µL dNTP (1 mM; MBI Fermentas, USA); 0.8 µL 25 mM $MgCl_2$; 5 U Taq DNA polymerase (MBI Fermentas, USA); and 3 µM of each primer. Primers used are listed in Table1.

**Table 1.** Mutation sites, primer sequences, annealing temperatures (Ta), PCR product sizes, and RFLP patterns of *MC1R*, *KIT*, *IGF2*, and *NR6A1*.

| Gene/SNP | Primer sequence | Ta (ºC) | Product size (bp) | Expected PCR-RFLP position patterns |
|---|---|---|---|---|
| *MC1R*[a] | EPIG1F: 5'-GCCCGTGCTTGGCCC-3' | 62 | 405 | *Hha*I (GG : 208, 100 and 76 bp) |
| c.727 G>A | EPIG3R: 5'-GACACGTATCGGTCCACGG-3' | | | and (AA: 298 and 107 bp) |
| c. 729 A>G | | | | *Bst*UI (AA:222 and 183 bp) and (GG: 405 bp) |
| *KIT*[b] | F: 5'-GTAAGGCCCAGATGTTCTTCCT-3' | 60 | 538 | *Bst*UI (GG: 249,199,75 and 15 bp) |
| g.276G>A | R: 5'-CAACCGGACCTACTGAAATGAC-3' | | | and (AA: 249,199 and 90 bp) |
| *IGF2*[c] | F: 5'-CACAGCAGGTGCTCCATCGG-3' | 63 | 336 | *Nci*I (GG : 308 and 28 bp) and |
| g.162G>C | R: 5'-GACAGGCTGTCATCCTGTGG-3' | | | (CC: 208, 100 and 28 bp) |
| *NR6A1*[d] | F: 5'-CATCCTCTTGCCTCCCTTAC-3' | 60 | 360 | *Nci*I (TT: 360 bp) and |
| g.99084751C>T | R: 5'-ATCCTGAGCACCCAGTCTAAC-3' | | | (CC:183 and 177 bp) |

[a]Primers for *MC1R* form Kijas et al. (1998), [b]*KIT* primer from Chung and Chung (2010), [c]*IGF2* from (Vykoukalova et al., 2006), and [d]*NR6A1* from Yang et al. (2009).

Amplifications were carried out in a T-Professional Standard Thermo cycler (Biometra GmbH, Gottingen, Germany) under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles of 45 s denaturation at 94°C, 30 s at annealing temperature (Table 1) and 45 s extension at 72°C; and, a final extension at 72°C for 5 min. Agarose gel electrophoresis was used to determine the size and quantity of PCR products.

## Restriction enzyme digestion and gel electrophoresis

The PCR products were digested in a total volume of 10 µL, consisting of 3 µL PCR products, 1 µL 10X reaction buffer, 1 U of each restriction enzyme (*Bst*UI, *Hha*I and *Nci*I), and the reaction was digested overnight at 60°C for *Bst*UI, and at 37°C for *Hha*I and *Nci*I enzymes (New England BioLabs, Hercules, USA). Digested products of *MC1R*, *IGF2*, and *NR6A1* were separated on 2% agarose gel by electrophoresis; PCR products of *KIT* required 6% non-denaturing polyacrylamide gel and visualized using Gel Star (Lonza, USA) for the detection of RFLP patterns between pig breeds (Table 1).

Amplification products of intron 19 of *KIT* gene were sequenced for each pig breed, using an Applied Biosystems 3730XL Genetic Analyzer (Applied Biosystems, USA) in 1st BASE DNA Sequencing Service. The aligned sequences were analyzed using the ClustalW2 software available online (http://www.ebi.ac.uk/Tools/msa/clustalo/).

## Data analysis

Allele and genotype frequencies were calculated for each breed and deviations from the Hardy-Weinberg equilibrium (HWE) between SNPs were tested using the GENEPOP software, version 4.2 available online (http://genepop.curtin.edu.au/) (Raymond and Rousset, 1995). Our study used a deterministic approach to look for markers of different allele variations fixed within different breeds. The development of simple analysis protocols was made possible without statistical inference (Dalvit et al., 2007).

## RESULTS

Polymorphisms in *MC1R*, *KIT*, *IGF 2* and *NR6A1* genes were detected using PCR-RFLP. We identified five nucleotide substitutions previously associated with coat color in pigs (c.727G>A

and c.729A>G in *MC1R*; g.276 G>A in *KIT*; g.162 G>C in *IGF2*; and g.299084751C>T in *NR6A1*). Additionally, we identified a new variation in intron 19 of *KIT* with a 14 bp deletion, which has not been previously reported. These mutations were analyzed in five pig breeds; a chi-square test showed that breed populations were not in Hardy-Weinberg equilibrium ($P \leq 0.05$).

## Genotype frequencies of *MC1R* and *KIT* in pigs

Genotyping was performed in various pig breeds, according to the RFLP pattern of digested PCR products (Table 1). The PCR-RFLP methodology employed produced expected restriction patterns for pig breeds; SNPs were detected at c.727G (enzyme *Hha*I) and c.729A (*Bst*UI enzyme) of the *MC1R* gene. The allele frequency of SNP at position c.727 (G>A), or codon 238 mutation analysis, showed that all DU were of the homozygous genotype AA (red in color), and belonged to a fixed, single breed. In contrast, the allele frequency of SNP at position c.729 (A>G) or codon 240 mutation of the *MC1R* gene, presented wild-type coat colors or homozygous genotypes (GG) in WB only, and was not detected in other pig breeds (Table 2).

**Table 2.** Genotype frequencies of *MC1R* and *KIT* mutations in four pig breeds.

| Breed | N | *MC1R* | | | | *KIT* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | c.727G>A | | c.729G>A | | g.276G>A | | 14-bp deletion | | |
| | | GG | AA | GG | AA | GG | AA | N/N | N/D | D/D |
| WB | 14 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| TNB | 35 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |
| TNBW | 20 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0.95 | 0.05 |
| DU | 35 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| MS | 25 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |

WB = Wild boar; TNB = Thai native (black coat color); TNBW = Thai native (black and white coat color); DU = Duroc; MS = Chinese Meishan.

The mutation at g.276G>A, in intron 19 of the *KIT* gene, resulted in RFLP patterns containing fragment sizes of 249, 199, 75, and 15 bp in WB, MS, and TNB pigs; and 249, 199, and 90 bp in DU pigs; this was in accordance with expected outcomes. However, unexpected patterns were seen in TNBW pigs; in which a new variation of intron 19 of *KIT* presented 249, 199, 185, 75, and 15 bp; and 249, 185, 75 and 15 bp (Figure 2A). The new presence of 185 bp in intron 19 of KIT was confirmed by sequencing analysis. Surprisingly, the 185-bp fragment resulted in the deletion of 14 bp in intron 19 (intron 19-g.98delAAAATGTCACTTGG; Figure 2B); TNBW pigs showed the heterozygous genotype for this site. Whereas the TNB and MS pig exhibits black coat color and WB have a wild-type coat no carriers of the 14 bp deletion in intron 19 of the *KIT* gene.

## Allele and genotype frequencies of *IGF2* and *NR6A1* in pigs

In our study of the SNP located in intron 7 of the *IGF2* gene (*IGF2*-in7-G162C), we anticipated that the G allele would be fixed in Thai native pigs. Although findings were in accordance with this hypothesis, TNB and TNBW pigs had high frequencies of the G allele (or homozygous genotype GG, and heterozygous genotype GC) but they were not fixed. High frequencies of the G allele were also seen in WB and DU pigs, while MS pigs had high frequencies of the C allele (or homozygous genotype CC and heterozygous GC; Table 3).

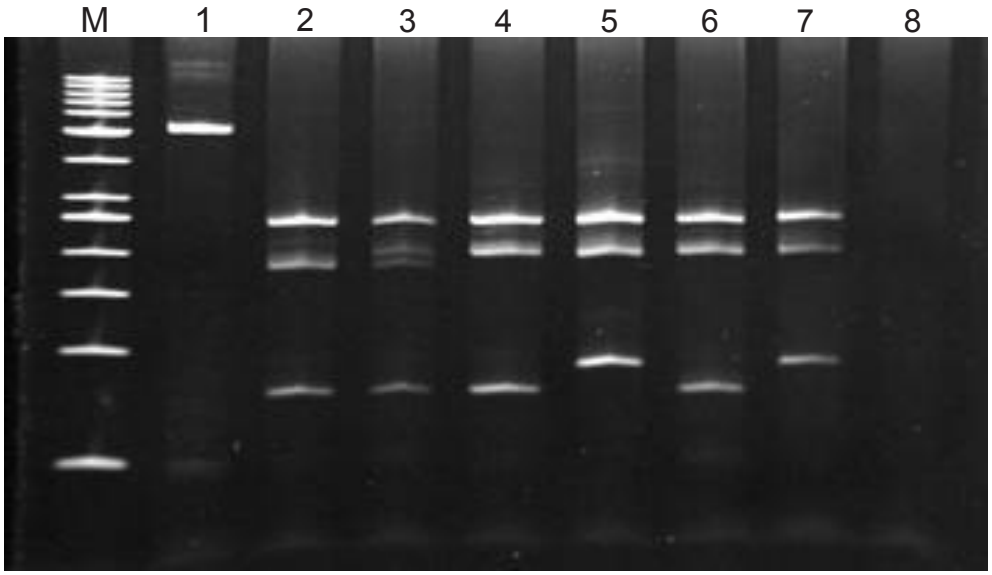


**Figure 2.** RFLP patterns of amplified 538-bp PCR product of the *KIT* gene generated using *Bst*UI: *line M* = ladder (50 bp); *lane 1* = PCR product. *Lanes 2-7* = digested products from different pig breeds: TNBW, with 14-bp deletion (*lanes 2-3*); TNB and WB (*lane 4*); DU (*lane 5*); MA (*lane 6*); and DU (*lane 7*). *Lanes 4-7* show no deletion. *Lane 8* = negative control.

For the observed mutation at g.299084751C>T within *NR6A1*, we found that the T allele, or homozygous genotype TT (associated with vertebrae number) was fixed in DU pigs. However, we found varying T and C alleles in TNB, TNBW and MS pigs, and the T allele was absent in WB pigs (Table 3).

**Table 3.** Genotype and allele frequencies of *IGF-2* and *NR6A1* in four pig breeds.

| Breed | N | *IGF2*/intron 7-G6162C | | | | | *NR6A1*/g. 299084751C>T | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | G | C | GG | GC | CC | T | C | TT | TC | CC |
| WB | 14 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| TNB | 35 | 0.70 | 0.30 | 0.57 | 0.27 | 0.16 | 0 | 1 | 0 | 0 | 1 |
| TNBW | 20 | 0.63 | 0.37 | 0.47 | 0.31 | 0.21 | 0.23 | 0.76 | 0.06 | 0.38 | 0.56 |
| DU | 35 | 0.17 | 0.83 | 0.07 | 0.20 | 0.73 | 1 | 0 | 1 | 0 | 0 |
| MS | 25 | 0.34 | 0.66 | 0.24 | 0.20 | 0.56 | 0.34 | 0.66 | 0.24 | 0.2 | 0.56 |

WB = Wild boar; TNB = Thai native (black coat color); TNBW = Thai native (black and white coat color); DU = Duroc; MS = Chinese Meishan.

## DISCUSSION

Functional genes regulate the predominance of specific traits. Identifying and disseminating purebred and crossbred pigs has become easily and reliably achieved using PCR-RFLP methods (Kijas et al., 1998; Fontanesi et al., 2014).

Mutations of the *MC1R* and *KIT* genes have also been used in the identification and traceability of local pig breeds in many countries (Babicz et al., 2013; Fontanesi et al., 2014). Kijas et al. (1998) characterized two mutations (p.M73K and p.D121N) determining the dominant black

($E^{D1}$) allele in large black and MS pig breeds. The presence of the $E^{D1}$ allele was also observed in other breeds, such as the black Slavonian pig (Margeta et al., 2009). Similarly, the genotypes of TNB and MS pigs also produce a dominant black $E^{D1}$ allele of *MC1R*. The $E^{D1}$ allele was suggested to be the major allele controlling the black coat color within local pig breeds (Kijas et al., 1998). The WB of Thailand showed the same $E^{+}$ allele as breeds of European and Asian WBs (Koutsogiannouli et al., 2010; Fontanesi et al., 2014). We may conclude that the $E^{+}$ allele is the major allele that controls coat color in WB worldwide. The SNP at position c.727 (G>A), associated with the red color (*e* allele) of the extension locus, proved to be similar within DU pigs (Fontanesi et al., 2014).

The *KIT* gene was found to control the dominant white color, as well as the white belts and black color in pigs. Chung and Chung (2010) showed that the presence of two alleles, g.276G and g.295A at intron 19 of *KIT*, produced a fixed black coat color in Korean native pigs as well as the g.276A allele in Landrace, DU, and Yorkshire pig breeds. The results in our study have shown the allele g.276G present in Thai native pigs, however not so with the variation g.295A. Interestingly, heterozygous forms of 14 bp deletions within the same region were observed in TNBW pigs. There have been no reports of this allele having an effect on coat color in pig breeds to date (Figure 2A and B). Although this polymorphism was analyzed in TNBW pigs only, further research on the effect of this novel allele on coat color is required and may lead to further development of traceability in the Thai native pig supply chain.

The *IGF2* gene was marked as a candidate gene influencing growth, meat quality, and fertility traits in pigs (Kolaríková et al., 2003; Muñoz et al., 2010). Vykoukalová et al. (2006) reported that Landrace, large white, and DU pigs had C allele with a high frequency and that the C allele was fixed in Hampshire pigs. Additionally, these authors reported a linkage disequilibrium between *IGF2*-in3-G3072A and *IGF2*-in7-G162C polymorphisms, in which (G-G/G-G) showed higher back fat than (A-C/A-C) genotypes ($P < 0.01$) in large white gilts (Vykoukalová et al., 2006). In our study, the G allele maintained high frequencies in Thai native and wild boar, whereas DU and MS pigs had a high frequency of the C allele. Fontanesi et al. (2010) and Alves et al. (2012) found that the *IGF2* intron 3-g.3072 G allele was fixed or almost fixed in local breeds (Cinta Senese, Casertana, Nero Siciliano and Iberian pig breeds), and present at high frequencies (IGF2g.3072A) in DU pigs. However, it is said that the fecundity of the MS breed is approximately three to four piglets per litter higher than that of western pig breeds. Muñoz et al. (2010) detected polymorphisms in a hyper-prolific Chinese-European pig line (Tai-Zumu) and found that the paternal allele *IGF2*-intron 3-3072A was associated with increased litter sizes. On the other hand, Yang et al. (2006) found that the genotype and allele frequency of *IGF2* 3072G>A in Chinese native pig breeds with GG genotype had a high frequency.

Variations in rib and vertebrae numbers usually influence body size, carcass length, and teat number in pigs (Mikawa et al., 2007; Duijvesteijn et al., 2014). Native pigs and wild boar have 19 vertebrae, whereas European commercial breeds for pig production have 20 to 23 vertebrae; this is due to selection for intensive pig production, which has increased the number of teats and litter size in pigs (Rubin et al., 2012; Duijvesteijn et al., 2014). Previous studies have reported that *NR6A1* and *VRTN* have a controlling influence on the number of vertebrae in pig breeds but the allele of *VRTN* is not fixed in any pig breed. In *NR6A1,* at 299084751, the mutation C>T is associated with an increased number of vertebrae, and appears to be fixed in European purebred and commercial crossbred pigs (Burgos et al., 2015). Although most native pig breeds have lower vertebral numbers than exotic breeds, we found that in the current study, the T allele varied in TNBW pigs. This was consistent with a reported body length of TNBW pigs of approximately 127 cm, longer than that of

TNB pigs, which have an approximate body length of 85 cm) (Rattanaronchart, 1994).

In many countries, the development and utilization of genetic resources in native pig breeds help to determine optimal breeding strategies but also have other outcomes, such as supporting cultural and social bonds, and providing extra income for small-scale farmers. Selective breeding for improved meat quality and reproduction characteristics in native pigs has resulted in the production of high quality meat products. Thailand, however, has yet to fully benefit from the genetic characterization and information of native pigs. To this end, genetic information on functional markers may elucidate differences in coat color, meat quality, and reproductive performance of Thai native pigs. Also, the difference of *MC1R* and *KIT* genotype and frequency among the Thai native, WB, and DU and MS pig breeds are the markers used to separate genetic differentiation and may be useful for conservation of endangered native pig in Thailand.

## Conflicts of interest

The authors declare no conflict of interest.

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