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Polymorphism in the Melanocortin-4 Receptor (*MC4R*) Gene and its Effect on Fatness and Weight Performance of Philippine Native Pigs: A Preliminary Study

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Abstract

The single nucleotide substitution (Asp298Asn) in the melanocortin-4 receptor (*MC4R)* gene has been reported to be associated with several production traits in different swine breeds. However, other related studies obtained inconsistent results in terms of its influence on the fatness and growth performance of pigs. This pilot study aimed to investigate single nucleotide polymorphism present in the *MC4R* gene, determine its frequency and its relationship to fatness and weight performance of the Philippine Native Pig (PNP). Genomic DNA was extracted from hair follicle of 86 PNPs of different ages and sexes and subsequent genotyping was done via restriction fragment length polymorphism (RFLP). The frequency of allele G (0.623) was higher than allele A (0.377) in the population sampled.

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The age (period of birth) affected the birth weight, adjusted 30-day weaning weight, average daily gain and backfat thickness (BFT) of pigs while the sex of the PNPs only had a significant effect on the BFT. However, polymorphism in the *MC4R* gene did not significantly influence the fatness and weight performance of the PNPs in this study. The no significant influence of *MC4R* genotype on several production traits in this study makes *MC4R* gene a questionable candidate gene for marker-assisted selection (MAS) in PNPs. Follow-up studies with larger sample size should be conducted to further elucidate the effect of the polymorphism on production traits of these indigenous swine in the Philippines.

Keywords: Asp298Asn; missense mutation; *MC4R* gene; Philippine Native Pig; RFLP

1. Introduction

Studies in the field of animal and veterinary sciences in the present are geared towards the molecular approach of studying biological organisms. To date, physiological mechanisms such as growth and metabolism can be much more understood at the molecular level. Candidate gene approaches which are widely employed recently have been successful in the identification of major genes affecting different traits in animals. One of the identified candidate genes for growth in pigs is the melanocortin-4 receptor (*MC4R*) gene [1]. Physically mapped to porcine chromosome 1, a single nucleotide substitution (G→A) in the *MC4R* gene within a *TaqI* restriction site was identified in swine of different breeds [2,3]. This polymorphism is a single missense mutation brought about by the replacement of aspartic acid (GAU) with asparagine (AAU) at the position identical to amino acid 298 (hence, Asp298Asn) of human *MC4R* protein within the seventh transmembrane domain [4]. Whereas Asp298 is required for normal signaling to adenylyl cyclase, the Asn298 variant could not stimulate cAMP production [5].

The *MC4R* gene is now a subject of research both in the fields of genetics and physiology. Biological studies have regarded *MC4R* gene to have an influence on the regulation of feeding behavior, body weight, energy homeostasis and hypothalamic-pituitary-adrenal (HPA) axis through certain physiological mechanisms in different species [6,4]. Thus, polymorphisms identified in the gene responsible for encoding the melanocortin-4 receptor protein are looked at as potential genetic markers for growth-related traits in pigs such as feed intake, growth rate and fatness. Several studies on *MC4R* gene in commercial swine breeds, Chinese pig breeds, Lithuanian White pig, Pulawska pig and wild boar were conducted [4, 6-11]. Although many studies revealed significant association of the missense mutation in the porcine *MC4R* gene with growth, fatness, carcass composition and meat quality in pigs [4, 7, 9, 11-14], inconsistencies in the results are evident in other studies conducted [15-19].

In the Philippines, the idea of conserving and utilizing native animals including the Philippine Native Pig (PNP) is gaining ground in agricultural production systems in response to the global concern on climate change. Concerted efforts on the conservation, evaluation and commercialization of the PNPs are now on board upon seeing the advantages of raising indigenous pigs i.e., big savings in the feed and housing costs, utilizing locally available feed materials, higher resistance to parasites and diseases, higher tolerance to cold and hot environments with emphasis in the warm climate zone and better adaptability to local conditions than imported

breeds. All these efforts are being done despite the disadvantages of raising PNPs due to their genetic limitations which include small body size, low feed conversion ratio, unpredictable production performance and product quality [20].

Considering the advantages of conserving and utilizing PNPs in livestock production systems in the Philippines and the claims on the effect of polymorphism in the porcine *MC4R* gene on several production traits, it is interesting to explore the relationship of the missense variant of the *MC4R* gene on the growth and fatness of PNPs. The potential for utilizing *MC4R* gene as candidate gene for growth and fatness could be of significance in an attempt to improve the performance of PNPs via marker-assisted selection. Hence, this preliminary study aimed to identify the single nucleotide polymorphisms present in the *MC4R* gene, determine its frequency and relationship to fatness and weight performance of the PNPs. This study was also conducted to investigate the effect of sex and age on several production traits in PNPs.

2. Materials and Methods

Experimental animals

Hair follicle samples were collected from a population of 86 Philippine Native Pigs of different ages (3-5 months, 6-9 months and 10-12 months) and of different sexes kept at the National Swine and Poultry Research Development Center (NSPRDC), Brgy. Lagalag, Tiaong, Quezon.

Hair follicle samples of PNPs were collected using tweezers or simple plucking by hand. Appropriate measures were observed to avoid contamination of the hair samples. The hair samples were placed in resealable plastic bags, carefully labeled and transported to the Animal Biotechnology Laboratory, Animal Breeding and Physiology Division, UPLB. The phenotypic data of the PNPs used in the study was provided by the center and the backfat thickness on the 10^{th} rib of the animals was measured using a pulsed ultrasound device - Renco Lean-Meater (*Renco Corp., Minneapolis USA*).

Genomic DNA extraction and PCR amplification of porcine *MC4R* gene fragment

The genomic DNA was isolated from the hair follicle of PNPs. The procedure for the extraction of DNA was done using SolGent Genomic DNA Prep Kit (Solution type, South Korea) with modifications of protocol indicated in the package manual. Hair follicles were cut and placed in a 1.5 mL microcentrifuge tube. One hundred (100) µL of lysis buffer was then added to the hair follicle samples and was vortexed for 20 seconds to ensure that the solution was properly mixed. The samples were then centrifuged at 10,000 rpm at 25°C for 1 minute. Samples were incubated at 55°C overnight. Post-incubation procedures include the addition of 50 µL protein precipitation solution to the sample, vortexing and centrifugation at 13,000 rpm at 25°C for 3 minutes. The liquid was then transferred into a new 1.5 mL microcentrifuge tube, followed by the addition of 150 µL isopropanol and subsequent centrifugation at 13,000 rpm, 25°C for 2 minutes. The liquid was then discarded and removed followed by the addition of 75% ethanol and subsequent centrifugation still at 13,000 rpm, room temperature for 2 minutes. The latter step was done twice before the samples were dried in the laminar flow cabinet. After drying, 20 µL of DNA hydration was added in the samples, mixed by pipetting and incubated in

the oven at 66°C for 1 hour. After extraction of the DNA, the genomic DNA was checked and analyzed by 1% agarose gel electrophoresis.

For the amplification of specific *MC4R* gene fragments, the following primers released by [4] were used: Forward: 5' – TAC CCT GAC CAT CTT GAT TG -3' and Reverse: 5' – ATA GCA ACA GAT GAT CTC TTT G -3'. The PCR conditions for the amplification of the porcine *MC4R* gene fragment is also described in the article published by [4]. The PCR components include 12.5 ng of genomic DNA, 1 X PCR buffer, 1.5 mM MgCl2, 0.125mM dNTPs, 0.2 µM of each primer and 0.35 U *Taq* DNA polymerase (*Vivantis Technologies, Malaysia*) in a 20 µL final volume. The components were subjected to the following cycling profile: 2 min at 94°C; 35 cycles of 30 s at 94°C, 1 min at 56°C, 1 min 30 s at 72°C; and a final 15-min extension at 72°C in a thermal cycler (*G-Storm, UK*). Gel documentation was done after subjecting the amplicons to electrophoresis on 2.5% agarose gels.

The PCR products of pigs homozygous for allele G and allele A were submitted to the DNA Core Sequencing Facility of the Philippine Genome Center, UP Diliman, Quezon City for capillary sequencing (single-pass reaction: forward and reverse). This was done to ensure that the amplified DNA sequence is indeed the porcine *MC4R* gene. Basic Local Alignment Search Tool (BLAST) was used to calculate the sequence similarity of the samples to the gene of interest.

Analyses of SNPs in the *MC4R* gene

Restriction Fragment Length Polymorphism (RFLP) method was used to detect polymorphisms in the *MC4R* gene of PNPs using *TaqI (Vivantis Technologies, Malaysia)* as the restriction enzyme. The reaction mix was placed in a 1.5 mL microcentrifuge tube which includes the reaction buffer, restriction endonuclease, DNA grade water and lastly the PCR product. Aliquots of 10 µL PCR products were digested with 5 units *TaqI* restriction endonuclease. The samples where then incubated at 65°C for 7 hours without thermal inactivation. The scoring and subsequent visualization of restriction fragments were carried out using gel electrophoresis on 3.5% agarose and were viewed using a gel documentation system (*Bio-Rad Laboratories, California, USA*).

Statistical analysis

In order to determine the relationship of *MC4R* genotype to the weight performance and backfat thickness of Philippine native pigs, a three-way factorial (3 X 3 X 2) in a completely randomized design (CRD) was used in the statistical analysis. The design used accounted for several factors which include the *MC4R* genotype, sex and age of the animals used in the study. However, significant interaction effects between the 3 different factors were not observed for all the parameters and therefore were no longer included in the full model. The mathematical model used in the study is as follows: $Y_{ijkl} = \mu + MC_i + S_j + A_k + e_{ijkl}$, where Y is the lth production traits of the native pigs (BW, A30dWW, ADG and BFT); µ is the average of population; MC is the ith effect of the porcine *MC4R* genotype; S is the jth sex of pig; A is the kth age of pig and e is the random error. The phenotypic data obtained were analyzed using the General Linear Model procedure (PROC GLM) of the Statistical Analysis Software package (*Cary, USA*). The computed values were expressed as least square means

(LSM) ± standard error (SE). The genotypic frequencies were determined by manual counting and allele frequencies were then computed.

3. Results and Discussions

The amplified PCR fragment for the analysis of the porcine *MC4R* gene polymorphism was about 226 bp long. A 50 bp- ladder was used in order to ensure that the size of the PCR product fall across the expected size when analyzed via gel electrophoresis on 2.5% agarose. The documented image of the gel of the amplicon is shown in Figure 1. Results of BLAST analysis of the PCR products subjected to capillary sequencing showed 98-100% sequence homology and is confirmed to be the *MC4R* gene of *Sus scrofa*.

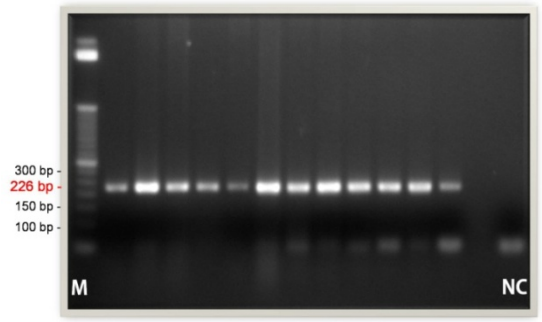

Figure 1. Agarose gel electrophoresis (2.5%) of specific porcine melanocortin receptor-4 226 bp-fragment produced via polymerase chain reaction procedure. (*M – 50bp marker)

After subjecting the product to digestion using *TaqI* restriction enzyme, allele G produced 156 and 70 bp fragments while allele A produced a lone 226 bp fragment. Meanwhile, both fragments of alleles A and G were found in the heterozygous genotype (see Figure 2).

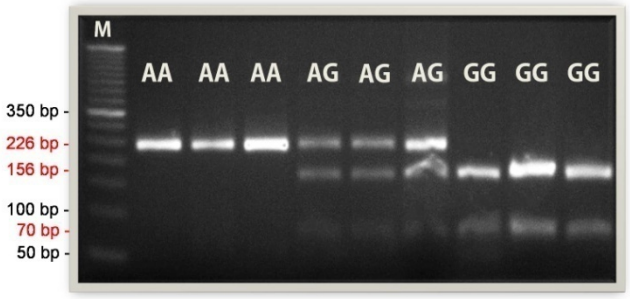

Figure 2. Agarose gel electrophoresis (3.5%) of digested PCR product using *TaqI* restriction enzyme. Lane 1: 50 bp molecular marker; Lanes 2-4: Allele A produced a 226-bp fragment; Lanes 8-10: Allele G produced 156 and 70-bp fragments Lanes 5-7: Heterozygote (AG) produced both fragments of alleles A and G.

The genotype and allele frequencies for the G→A substitution of the *MC4R* gene in Philippine Native pigs are shown in Table 1. The frequency of allele G in a population of indigenous pigs was higher than that of allele A. Animals with the genotype AG had the highest frequency, followed by pigs homozygous for allele G. On the other hand, genotype AA had the lowest frequency in the sampled population of native pigs. The allele frequencies of PNPs obtained in this study is similar to several *MC4R* genotype analyses in other studies

involving different swine breeds. Higher frequency of allele G was also observed in a group of pigs of different breeds ranging from crosses of established commercial swine breeds i.e. Landrace, Large White, Pietrain, Duroc, Hampshire reared in different countries as well as local Chinese pig breeds i.e., Erhualian, Jiangquhai, Neijan, etc. and Italian local breeds i.e., Cinta Senese, Calabrese and Casertana [11, 15, 16, 18]. In contrast, high allele A frequency was observed in other studies using synthetic breeds, Chinese local breeds i.e. Xiang and Guangdong Large Black White and cosmopolitan breeds in other countries [3, 4].

Table 1. Genotypic and allelic frequencies of the *MC4R* gene in Philippine Native Pigs (n=86)

Genotypic Frequency (%)		Allele Frequency (%)	
GG	40.9	G	62.3
AG	42.7	A	37.7
AA	16.4		

The effects of the porcine *MC4R* genotypes, age, and sex on different production traits of the PNPs are presented in Table 2. Numerical differences on the effects of the genotypes on ADG as well as the BFT were observed. Pigs homozygous for allele A had the heaviest BW but had the lowest ADG and BFT whereas pigs heterozygous for both the *MC4R* alleles G and A had the lowest BW but highest ADG. Highest BFT value was recorded in pigs with the GG genotype. Although numerical differences were observed on the several production traits of the pigs with different *MC4R* genotypes, the effect of the *MC4R* gene polymorphism on the BW, A30dWW, ADG and BFT were not statistically significant. Many studies demonstrated the association of porcine *MC4R* single nucleotide polymorphisms with growth, fatness, carcass composition and meat quality in different swine breeds [4, 7, 9, 11-14]. Other studies had different results in that only some of the production traits turned out to be influenced by the single nucleotide polymorphism in the porcine *MC4R* gene. For instance, [18] showed that only fatness but not growth rate was associated with the missense mutation in the gene. In a study conducted by [16], the significant effect of the *MC4R* genotype on fatness was only observed in pigs slaughtered at high live weight but not in pigs slaughtered at low live weight.

Whereas the aforementioned studies revealed the significant influence of the Asp298Asn polymorphism of the *MC4R* gene on fatness and growth performance in pigs, other related studies found no significant effect of the said genotype on the weight performance and fatness in pigs in which the results of this study coincided. The spinal fat thickness between Large White pigs with different *MC4R* genotypes was not significantly different from each other [19]. In a small sample of pigs harvested, the effect of the SNP in the gene on the ultrasonically measured 10^{th} rib backfat was found to be not significant [17]. Furthermore, [15] did not find any evidence on the effect of *MC4R* missense G-A SNP on the daily food intake, BFT or abdominal fat. It is clear that previous studies on the effect of the *MC4R* genotype on the several production traits obtained varying outcomes. In this study, all the production traits were not affected by the *MC4R* genotype of the PNPs and seemed to have agreed with the results obtained in the previous studies [15, 17, 19].

In addition the results of the present study is also different from previous studies in that even if there were no significant differences recorded, the numerical values obtained in several production traits of PNPs showed a

different trend compared to results in different swine breeds [4, 7, 11, 16, 21]. Results in studies cited above claimed that allele A in the Asp298Asn missense mutation in the porcine *MC4R* gene is significantly associated with faster growth and thicker backfat. However, in this study, lower numerical values were observed in the A30dWW, ADG and BFT in PNPs homozygous for allele A than pigs with both the GG and AG genotypes. In fact, the growth rate may have been hampered by low numerical ADG value recorded in these pigs since these animals, although not significant, had the highest recorded BW. In a study by [4], one of the lines, termed line E, which was a cross between Chinese (Meishan) breed and a line with a Large White origin, had an opposite result compared to lines A (Landrace), B (Large White), C (synthetic between Large White and Duroc populations) and D (synthetic line derived from several different synthetic populations). It was noted that allele G, although not significant, was associated with the fattest animals in line E opposite from its counterparts in other lines. Possibilities reported include linkage disequilibrium between the mutation and the causative mutation and difference in the background gene effects (epistasis) since traits for growth and fatness are governed by many genes (polygenic) and are considered to be complex [4]. In addition, [22] revealed that the effect of *MC4R* gene on the production traits in pigs may depend on other SNPs or the interaction of several SNPs in the entire porcine *MC4R* gene i.e., backfat and growth values were influenced by the p.Arg236His while p.Asp298Asn primarily influenced the variation in growth rate of the population. Moreover, there was a significant interaction that was observed between the two above cited SNPs in terms of ADG.

In this study, it is also possible that the PNPs have a different genetic background and do possess distinct allelic interactions compared to other swine breeds used in previous studies. The research center has been maintaining native pigs coming from the provinces of Benguet, Marinduque, Quezon and Kalinga since the late 90's and these pigs were carefully selected, bred and developed to improve their performance. Interventions made for the improvement of the population may have caused the alleles to interact differently or it may be inherently unique to the PNPs. In addition, the nutrition of PNPs in the farm might have also impeded the full manifestation of the genetic influence of *MC4R* on their production traits. Unlike PNPs, commercial breeds of swine are given feeds of high quality that are well-formulated and their diets are carefully monitored. Since phenotype is dependent on the gene, environment as well as the interaction of both (P = G + E + G X E), the feeding practices in the farm and the quality of feeds ingested by the PNPs are not ruled out as one of the causes of the discrepancy in the results. To date, the cause is unknown and has to be investigated further.

One of the limitations of this study is the small number of animals used in the experiment. Majority of the native pigs in the Philippines are raised in the backyard and thus managed differently. In order to avoid errors brought about by different management practices and conditions of the PNPs, the PNPs used in the relationship study were all from the NSPRDC. The small sample size of PNPs used may have also caused the insignificant negative association results in this study. A study by [9] showed that reduced number of animals may have caused negative association results in Meishan x Large White and Wild Boar x Large White intercross brought by the inability to detect small sized effects. Hence, in this study, the limited sample size of the PNPs was not ruled out as one of the possible causes of the negative association results obtained.

In terms of age of the PNPs used in the present study, the BW was highest in pigs at 3-5 months and the lowest

BW was recorded in pigs at 10-12 months (P<0.01). In their A30dWW, however, the highest value was recorded in pigs at 10-12 mos. while the lowest A30dWW was observed in PNPs at 3-5 mos. The same result was obtained in terms of the ADG of the PNPs as ADG was seen to be highest in the sampled animals at 10-12 months and lowest in pigs at 3-5 months (p<0.01).

Table 2. Effects of *MC4R* genotypes, age and sex on different production traits of the Philippine Native Pigs

MC4R	**PRODUCTION TRAITS**[1] (LSM ± SE)			
GENOTYPE	BW (kg)	A30dWW (kg)	ADG (g)	BFT (mm)
GG	0.72 ± 0.02	3.43 ± 0.14	90.25 ± 4.66	9.55 ± 0.67
AG	0.71 ± 0.02	3.52 ± 0.16	93.62 ± 5.05	9.52 ± 0.73
AA	0.76 ± 0.04	3.14 ± 0.27	79.39 ± 8.69	8.35 ± 1.25
AGE (mos)				
3-5	0.88 ± 0.02^{a}	3.05 ± 0.17^{b}	72.41 ± 5.38^{b}	4.71 ± 0.78^{c}
6-9	0.74 ± 0.04^{b}	3.33 ± 0.26^{ab}	86.30 ± 8.35^{ab}	9.49 ± 1.20^{b}
10-12	0.57 ± 0.02^{c}	3.70 ± 0.15^{a}	104.55 ± 4.72^{a}	13.24 ± 0.68^{a}
SEX				
Male	0.77 ± 0.03	3.46 ± 0.18	90.23 ± 5.64	7.66 ± 0.81^{b}
Female	0.70 ± 0.02	3.26 ± 0.14	85.28 ± 4.55	10.63 ± 0.66^{a}

[1] BW = birth weight; A30dWW = adjusted 30-day weaning weight; ADG = average daily gain; and BFT = backfat thickness

[a,b,c] means with different superscript within a column are significantly different

Expectedly, BFT was highest in pigs at 10-12 months, followed by animals at 6-9 months and lastly, PNPs at 3-5 months (P<0.01). It should be noted that the computation for ADG for all the animals used in the study was similar and not based on the age of the animals when the hair samples were taken. Thus, the formula used for determining their ADG was (A30dWW - Initial weight) / 30 days. Therefore, the significant influence as reflected by age (in the table) on the ADG of PNPs is a function of the period when the animals were born and not a function of the age per se. The period when the PNPs at 10-12 mos of age during the sampling may have been favorable for the animals to gain heavier weight at a certain period of time than that of the PNPs born in later periods. Meanwhile, the BFT was taken on site during the sampling proper of hair follicles of PNPs. The significant influence of age / period of birth on the Ad30dWW of the animals in this study can be attributed to the average daily gain of the PNPs since values of which were also significantly different from each other after being subjected to statistical analysis. The effect of the age / period of birth on the weight performance of the animals may be due to external factors such as environmental condition/season, feeds and management practices employed in the experimental farm between different periods.

As presented in Table 2, the sex of the animals did not significantly affect their BW, A30dWW and ADG. Numerical differences were observed especially in the ADG of the animals wherein values were higher in male

pigs than the female pigs. The difference, however, between the BFT of pigs as affected by sex was highly significant. Based on the result, female PNPs tend to deposit more backfat than the male animals even though the male pigs had numerically higher ADG than the females. Similar results were obtained in previous studies comparing the backfat layers of male and female swine. Female Gottingen minipigs had thicker relative backfat layers than males according to [23]. Moreover, it was reported that female Gottingen pigs became obese after *ad libitum* feeding while the males did not. Backfat depth was also 1.2 mm lower for boars than gilts confirming that gilts were significantly fatter at a similar weight and age than boars [24]. Likewise, [25] concluded that average fat depth was higher in female than male pigs.

4. Conclusion

This study provided preliminary results on the genotype and allele frequencies of the *MC4R* gene in PNPs. Allele G was observed to be twice as frequent as that of allele A. The age (period of birth) affected the BW, A30dWW, ADG and BFT of pigs while the sex of the PNPs only had a significant effect on the BFT. The no significant influence of *MC4R* genotype in animals used in this study makes *MC4R* gene a questionable candidate gene for marker-assisted selection (MAS) in PNPs. However, follow-up studies using larger sample size is highly recommended to confirm the effect of this gene on the growth and fatness traits in PNPs. Establishment of a breeding population of purified strains of native pigs is also suggested in order to obtain much more reliable results. Nevertheless, this study provided baseline information for future extensive *MC4R* gene research on native pigs in the Philippines.

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