Research Article

*ESR*1 Candidate Marker Gene Associated with Litter Size in the Philippine Native Pigs (*Sus philippensis*)

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Abstract | Improving the sow's prolificacy was one of the major concerns of the swine industry. Genes associated with litter size are widely reported and studied due to their economic impact. Estrogen receptor 1 (*ESR*1) gene has been linked to being one of the major genes affecting litter size in swine. The Philippine swine breed is not commonly used on a commercial scale but in backyard production. The aim of the study was to assess the allelic variation in 30 Philippine native pigs (PNP). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was done to evaluate the genotypes of *ESR*1 gene. Target gene was amplified and then subjected to RFLP using *Pvu*II restriction enzyme. Results showed that all of the samples were found to be of AA genotype which is not the favorable genotype according to previous studies. The litter sizes of the PNP were also determined. Results showed that the genotype of *ESR*1 gene present in PNP is homozygote AA. The average litter size of the 30 PNP is 7.07 which is relatively higher than the average litter sizes of Nepal native pigs which ranges from 2 to 6. Moreover, the average litter size of the Philippine breed is also higher than the 5.0 average litter size of Tibetan pig breed which is one of the common breeds of pigs for commercial pig production.

Keywords | Genotype, *Sus philippensis*, *ESR*1 gene, *Pvu*II, Philippines

Received | March 01, 2022; **Accepted** | May 09, 2022; **Published** | July 14, 2022
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Citation | Tabon GR, Medina NP, Uy-De Guia MRD, Mingala CN (2022). *ESR*1 Candidate Marker Gene Associated with Litter Size in the Philippine Native Pigs (*Sus philippensis*). Adv. Anim. Vet. Sci. 10(8):1747-1751.
DOI | https://dx.doi.org/10.17582/journal.aavs/2022/10.8.1747.1751
ISSN (Online) | 2307-8316

INTRODUCTION

The swine production in the Philippines including backyard production plays a major role in the animal industry. Needless to say, efforts in increasing the Philippine swine breed or native pig population is of major interest of animal breeders and scientists in the country. Considering the significance of native pigs as a genetic resource, therefore the need to analyze at the molecular level to maintain the animal genetic diversity and support the swine backyard production (FAO, 2009).

The swine industry is largest among the livestock and poultry industries of the country amounting to P191-billion (DOST-PCAARRD, 2016). It ranks next to rice with 18.28% contribution to the total value of agricultural

production and plays a major role in ensuring food security by providing about 60% of the total animal meat consumption of Filipinos. The Philippine swine industry is ranked eighth in the world in terms of the volume of pork production and number of breeding sows at the commercial scale. Backyard pork production which includes native pigs, has a cultural significance in the Cordilleras, the northern mountainous communities of Luzon island in the Philippines. Pork derived from native pig are often used during religious practices and rituals, historical depth, religious legend, and supernatural tradition (Cawed, 1972).

This research was carried out to determine the genotypes of the Philippine native pigs (PNP) (*Sus philippensis*) using Estrogen receptor 1 (*ESR*1) gene associated with litter size. According to Chen et al. (2000), litter size is one of the most important economic traits in pig production, and the more piglet numbers per litter, the more pork production and the more economic profit for pig industry will be achieved. While litter size is a trait which can be utilized to increase population of pigs, this trait has a low heritability. This therefore requires the need to study this gene for litter size in pigs to generate results which, according to Rothschild et al. (1996), could be utilized in the implementation of related marker-assisted selection programs to improve reproductive efficiency. Accordingly, the identification of individual genes or anonymous genetic markers associated with litter size in the pig could have a great economic impact on the swine industry. Omelka et al. (2005) also recommended the identification of genes or genetic markers associated with reproductive traits in pigs which could have a great economic impact on pork production. *ESR* gene is the major genes affecting phenotype of litter size without any genetic negative correlation to growth and carcass traits (Suwanasopee, 2011; Chen et al., 2000; Rothschild et al., 1996).

MATERIALS AND METHODS

Experimental animals

A total of thirty (30) PNP were randomly selected at Benguet State University Animal Genetic Resources in Benguet Province, the Philippines as experimental animals in this study. The age of the sample animals ranged from one to three years old.

Sample collection

A 5 mL blood was collected from the auricular vein of the animals using anticoagulated vial. Samples were placed in a cooler. Blood samples that were not processed immediately were refrigerated and were processed the following day. The blood collection protocol adhered to the IACUC for animal collection.

RNA extraction

Total RNA was extracted from the blood collected heparinized tubes for each sample with the use of TRIzol™ reagent (ThermoFisher Scientific, Massachusetts, USA). In a 1.5 mL MCT, 1000 µL of NH_4Cl and 500 µL of buffy/whole blood was mixed by pipetting, vortexed then centrifuged at 14000 rpm for 1 min and the supernatant discarded. The procedure was done twice until a white pellet was observed. 1000 µL TRIzol™ (Thermo Fisher Scientific, Massachusetts, USA) was added to the pellet and vortexed until mixture was homogenized. 200 µL chloroform was then added and vortexed then centrifuged at 14000 rpm for 10-15 min in a refrigerated centrifuge. The resulting supernatant was transferred to a new MCT containing 500 µL isopropanol. It was incubated for 10 min and centrifuged at 1400 rpm for 10-15 min in refrigerated centrifuge at 4°C. The supernatant was discarded and 500 µL of 75% ethanol was added to the pellet. It was mixed by inverting the tube and centrifuged at 14000 rpm for 5 min. Afterwards, supernatant was discarded and air dried under biosafety cabinet. Also, 50 µL of RNAse free water was added to rehydrate the pellet and stored at -80°C. The purity of the RNA extracts was evaluated by electrophoresis in 2% agarose gel.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT- PCR kit (SensiFAST™) was used to synthesize the cDNAs from the total RNA extracted. Two µL of dT random primers was mixed with 0.5 µL dNTP, 4.5 µL of RNAse free water and 3 µL of RNA template. The solution was incubated for 5 min at 65°C before running PCR. The prepared RNA primer mix was mixed with 2 µL 5x buffer, 0.5 µL reverse trancriptase, and 4.5 µL RNAse free water. This was subjected to PCR run for segment 1, 10 min for 30°C, segment 2, 45 min for 50°C, and segment 3, 5 min at 95°C. The product was also subjected to housekeeping gene (β- actin) check to assure of DNA extraction.

Restriction fragment length polymorphism (RFLP)

The genotype of *ESR*1 gene in PNP was determined the by using PCR-RFLP with the use of *Pvu*II restriction enzyme. The PCR-RFLP, which was based on the previous study was utilized and used the forward primer 5'-CCT-GTTTTTACAGTGACTTTTACAGAG-3' and reverse primer 5'-CACTTCGAGGGTCAGTCCAATTAG-3' and amplified an intron region of the *ESR* 1 gene located at pig chromosome 1 p.2.4 –p.2.5 (Short et al., 1997). The product was then digested with the *Pvu*II restriction enzyme which cuts CAG/CTG and resulted in a product of 120 base pairs.

The mixture contained 2 µL PCR products, 1 µL digestion

buffer and 0.3 µL of restriction enzyme. Afterwards, the mixture was incubated for 4h at 37°C. After the digestion, observation of formed restriction fragments of different sizes produced from the mixture and fragment (allele) identification were done by separation using gel electrophoresis. Fragments were separated on 2% agarose gels and visualized with Gel Red staining to view different fragment size and compare bands to the marker.

Frequency distribution of the litter size

Data on the litter sizes of the samples and frequency or count of the occurrences of values within a particular group or interval was counted and recorded (Table 1).

Table 1: Frequency distribution of the litter size.

Litter size	Frequency (n=30)	Percentage
5	2	6.67
6	9	30.00
7	9	30.00
8	6	20.00
9	3	10.00
10	1	3.33

RESULTS AND DISCUSSION

RT-PCR

The *ESR*1 gene was amplified from all of the cDNA samples with amplicon size of 120 base pairs as shown in Figure 1. PCR products were used for the RFLP analysis using *Pvu*II restriction enzyme. Due to presence of bands for all of the samples, the target region of *ESR*1 gene is present in PNP.

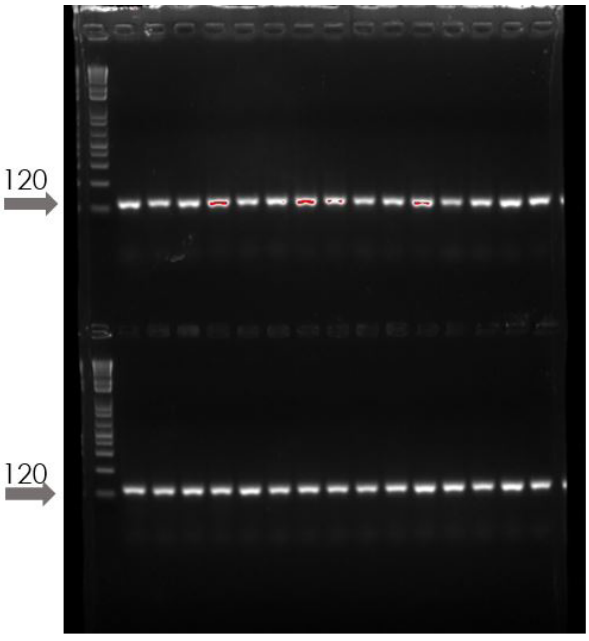

Figure 1: PCR amplification of 30 PNP targeting *ESR*1 gene at 120 bp using 100 bp ladder.

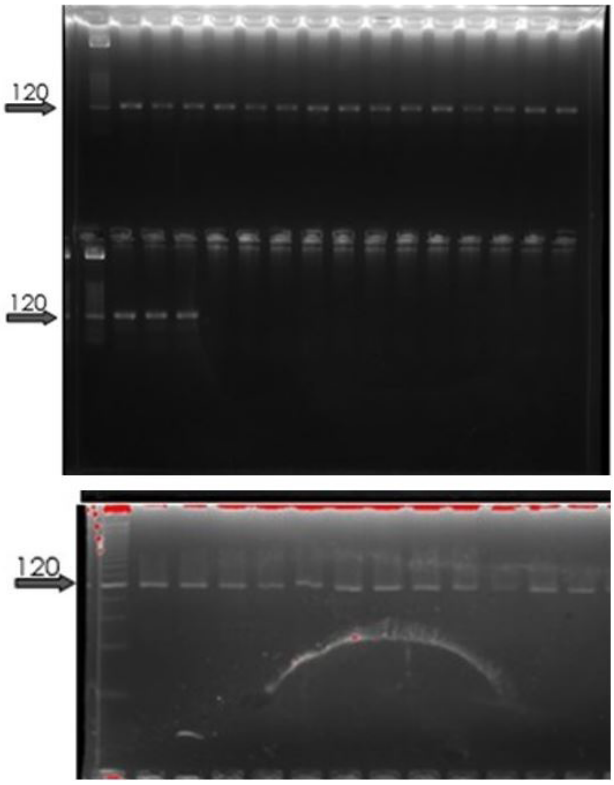

Figure 2: RFLP gel image of *ESR*1 gene in PNP using *Pvu*II restriction enzyme. Fragment size of all samples is 120 bp (genotype AA).

RFLP

RFLP analysis of *ESR*1 gene using *Pvu*II showed only one band pattern among all samples (Figure 2). Results showed that all samples are of AA genotype (one band at 120 bp) according to the study of Rothschild et al. (1996).

Frequency distribution of the litter size

The frequency distribution of the litter size of PNP is shown Table 1. It can be noted that both litter sizes of 6 and 7 has the highest frequency percentage of 30%. The litter sizes of 8, 9, 5, and 10 have lower frequency percentages of 20 %, 10%, 6.67 %, and 3.33 % respectively. The results of this study also showed that the average litter size of the PNP with genotype AA is 7.07. This value is higher than than the 5.22 average litter size of the PNP (Maddul, 1991) and the Nepal native pigs average litter sizes (FAO, 2009) which are 4-6, 2-6, 5.14, and 4.7 for Jangali Bandel; Pygmi Bandel; Hurra; and Bampudke, respectively. The 7.07 average litter size of the PNP is also higher than the average litter size of the Tibetan pig which is 5.0.

Native pig breed is a large group of native breeds which generally have poor production and reproduction performance levels (Legault, 1985) but are well adapted to their particular environment. In this study, *ESR*1 gene was examined as genetic marker for litter size in Philippine native pigs. The target *ESR*1 gene was successfully amplified in 30 PNP samples. However, only one band pattern was

observed during RFLP analysis which is the AA genotype (single band at 120 bp). This only indicates that the cutting site (5'-CAGCTG-3') for *Pvu*II is not present on the same region in PNP of the *ESR*1 gene. Studies on pigs showed that genotype AA is the most common genotype. The genotype BB has low rate of heritability even in pigs, hence the result of this study revealed genotype AA in 30 samples. These findings corroborate with previous studies on other pig breeds that showed a very low frequency of B allele in Polish Landrace (Kmiec et al., 2002) without *Pvu*II polymorphism (Drögemüller et al., 2001) Also, in the absence of reliable historical data on PNP, therefore further studies on Philippine pig populations such as DNA sequence analysis and examining the genotypic frequencies must be carried out to confirm the results of the present study.

The genotype of the of the *ESR*1, which is the part of the genetic makeup of the PNP, determines the expression of its phenotypic trait on litter size. According to Vicencio et al. (2017), litter size is one of the factors which is very essential in measuring reproductive success of a sow in swine operations. Chen et al. (2000) reported that litter size is one of the most important economic traits in pig production since more piglet numbers per litter increase pork production and bring more economic profit for the pig industry.

A comparison of litter size between PNP and other native pigs in Nepal and Tibet. Data showed that PNP had higher number of litter size than the other native pigs used in native pig production (FAO, 2009). Meanwhile, comparison between commercial breeds showed an average litter size of 7.07 for the PNP is lower than the average litter sizes of Yorkshire, Landrace, Hampshire, Duroc, and Meishan breeds which are 12, 11, 9, 9, and 14-17, respectively (FAO, 2009). Pietrain pig breed, which is one of the common breeds of pigs for commercial pig production, had a higher average litter size of 10.55 (Lukac et al., 2014) as compared to the average litter size of the PNP.

Based on the results, there exist differences among the different breeds of pigs in terms of litter size trait and this could be attributed to differences between pig breeds as reported by several authors. Litter size is a quantitative character of considerable complexity (Lukac et al., 2014) and the influence of environmental factors on its expression is significantly expressed (Wahner and Brussow, 2009). It is apparent that the background genetics of each different line plays an important role in the manner and magnitude of genetic control of this trait (Omelka et al., 2005).

An improvement of economically important traits including reproductive ones by selection is in the centre of interest within breeding programs all over the world (Bidanel et al., 1994; Li et al., 2004). Due to low heritability the use of genetic markers associated with reproductive traits is often applied to increase rates of genetic response and bring more economic profit to pig industry (Omelka et al., 2001; Buske et al., 2006). In the Philippines, where backyard production for Philippine native pigs plays a major role in the animal industry, utilization of gene markers for litter size can therefore accelerate the breeding programs implemented in the country.

CONCLUSIONS AND RECOMMENDATIONS

Based on the results of this study, the absence of the cutting site (5'-CAGCTG-3') in the amplified region (120 bp), showed that 30 samples were of AA genotype. This result could possibly be due to small sample size. The genotype identified for *ESR*1 gene in PNP is AA which was found to have an average litter size of 7.07. This baseline data on *ESR*1 gene can contribute to potential marker-assisted selection of native pigs to improve their reproductive traits, hence this research can be followed up with thorough verification of important regions in the gene that can be associated with litter size.

ACKNOWLEDGEMENT

We thank the Benguet State University for the support and the Philippine Carabao Center staff for the technical assistance to finish the study. Special thanks to the College of Agriculture, Central Luzon State University, Philippines.

NOVELTY STATEMENT

This study assessed the allelic variation of the PNP ESR1 gene. The result of this study suggested that the ESR1 gene can be used as a potential marker for assisted selection of native pigs to improve their reproductive traits, particularly the litter size.

AUTHOR'S CONTRIBUTION

Genevieve R. Tabon and Mary Rose D. Uy-De Guia performed the research activity, compiled the data and prepared the manuscript. Genevieve R. Tabon, Mary Rose D. Uy-De Guia, Noraine P. Medina and Claro N. Mingala designed the experiment, analyzed the data and prepared the manuscript. Claro N. Mingala for the final approval of the manuscript for publication. All authors have read and approved the manuscript before publication.

Conflict of interest

The authors declare no conflict of interest.

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