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ANALYZING RYR-1 GENOTYPE POLYMORPHISM IN MONG CAI STOCK PIGS AND THEIR F1 GENERATION AND DESINGING A KIT FOR DETERMINING THIS GENOTYPE IN PIGS

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Abstract. The Ryanodine Receptor-1 gene (RYR-1) is located in the 6p11 $\rightarrow$ q21 region on the No. 6 chromosome of pigs. RYR-1 is being investigated in several studies as a potentially important gene that is an indicator of pork quality. In this article, PCR-RFLP was used to analyze RYR-1 polymorphism in 19 Mong Cai pigs, 2 Yorkshire pigs and their 64 F1 generation pigs. The DNA fragment of 656 bp of RYR-1 was amplified using PCR that employs specific primers and is then digested by Hin6I. The results showed that Mong Cai pigs had only the homozygote *NN* (100%) at the RYR-1 locus while imported Yorkshire pigs had both the homozygote *NN* and the heterozygote *Nn* genotypes. Of their F1 generation, five out of 64 pigs were of the *Nn* genotype, and the others were of the *NN* genotype. We designed and developed a Master Mix kit set that can be used to identify RYR-1 in pigs' DNA genome. This kit can be used by breeding farms and for breeding programs. The kit can be used to quickly determine the RYR-1 genotype of pigs. The kit is very accurate and the measurements obtained are very reliable.
Keywords: Ryanodine Receptor-1 gene (RYR-1), porcine stress syndrome (PSS), malignant hyperthermia disease (MH), PCR-RFLP.

1. Introduction

In Vietnam, conventional pig breeding methods are the norm in agricultural production. There exist various local breeds of pigs and they are well adapted to the local

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environment, produce high quality pork and convert feed to meat economically. Local farmers have long used traditional methods of animal rearing and they have generally been unbable to selectively breed their limited number of animals for qualitative traits. The development of genomics and molecular genetics has made it possible to identify genetic linkage and a physical map of the pig genome. The application of molecular genetic markers and marker assisted selection (MAS) can improve the accuracy and reliability of pig breeding programs.

One of the loci which is useful in identifying pork quality is Ryanodine Receptor-1 (RYR-1). This locus encodes the channel which releases calcium ions on the reticulated membrane of bone's cells. The RYR-1 locus is located in the 6p11 $\rightarrow$ q21 region on chromosome 6 in pigs [3, 4, 7]. There is a single base-pair substitution mutation at +1843 position of this gene and it is called recessive allele *n* [2, 5, 6]. Pigs that have homozygous recessive genotype *nn* are susceptible to malignant hyperthermia (MH) disease and they are also liable to be affected by stress (porcine stress syndrome - PSS) [5]. Unfortunately, while pigs that have PSS disease have a high muscle to fat ratio, the meat is pale, soft and has excessive fluidity. In contrast, homozygous dominant genotype pigs (*NN*) have lean belly meat and thick back fat, but the overall quality of the pork is high. Heterozygous genotype pigs (*Nn*) have a comparative advantage over the two above genotypes in that they produce lean, good tasting pork and they are more PSS disease resistant [9]. Therefore, in selective breeding programs, a determination of the RYR-1 genotype if one wishes to market pigs that have leaner meat and a quality of meat that is acceptable.

In this study, we analyzed the genetic diversity of the RYR-1 locus in local pigs (Mong Cai) and imported pigs (Yorkshire) and also their F1 population.

2. Content

2.1. Materials and methods

2.1.1. Samples collection and DNA extraction

The 85 ear tissue samples of pigs used in this study were obtained from Lao Cai Province, Vietnam. The samples obtained were from Yorkshire pigs (n = 2), Mong Cai pigs (n = 19) and their F1 population (n = 64).

Genomic DNA was extracted from ear pigs tissue samples using the method described by Ausubel [1] which is as follows: 20 mg of ear tissue is frozen with liquid nitrogen and then ground to a fine powder consistency. Protease K is mixed into the powdered ear tissue and kept at 56^0C for 12h. It's then mixed with RNase and kept at 37^0C for 1h30'. Protein was precipitated in the presence of 7.5 M CH_3COONH_4 at -20^0C for 2h and then centrifuged, collecting the fluid. DNA was precipitated by mixing the fluid in isopropanol for 12h, then centrifuging that and collecting the precipitate. The DNA precipitate was washed in 70% alcohol and the resulting DNA was dissolved using

TE buffer and stored at 4^0C.

2.1.2. PCR and analysis of RYR-1 gene

Specific primers were designed to amplify the RYR-1 gene fragments, F: 5'-GTTTGCCACAGGTCCTACCA-3' and R: 5'-ATTCACCGGAGTGGAGTCTC-3'. Primers were purchased from the Roth Company.

PCR reactions yielded 50 - 100 mg of genomic DNA with 0.25 μM used in each primer in addition to 10X PCR of buffer, 0.5 μM of dNTPs and 0.5 U of Taq polymerase. PCR reactions were performed at 94^0C for 3 min, 35 cycles (94^0C for 45 sec, 64^0C for 1 min and 72^0C for 1 min) with an extension at 72^0C for 10 min. The PCR end product was stored at 4^0C.

PCR products were kept with restriction enzyme Hin6I (restriction site: G/CGC) at a ratio of 20 μl PCR product: 5 U enzyme Hin6I: 2.5 μl 10X buffer at 37^0C for 8h.

The products increasingly refined through the PCR process were subjected to gel electrophoresis (1% and 2% agarose/1X TAE buffer, respectively) and made visible using ethidium bromide stain.

2.1.3. Designing and testing a Master Mix kit to determine the RYR-1 genotype in pigs

** Design of the kit*

The Master Mix kit was designed according to the above PCR protocol and initiates a fragmentation reaction using a restriction enzyme. It includes 2 parts:

- PCR Master Mix kit: *Taq* DNA Polymerase (5U/μl), 10X *Taq* buffer (+KCl), 25 mM $MgCl_2$, 2.5 mM dNTPs and 10 pM of each primer for RYR-1 gene fragment amplification.

- Restriction enzyme kit: 10X buffer, enzyme Hin6I (10U/μl).

** Testing the kit*

The Master Mix kit was tested with concern for 2 criteria: stability and accuracy.

- Stability: the kit is used to analyze 35 DNA samples, 3 times repeated.

- Sensitivity: the kit is used to amplify the RYR-1 gene fragment of DNA samples that are diluted with different concentrations from 0 to 4 (μg/25μl), 3 times repeated.

2.2. Results and discussion

2.2.1. Amplification and analysis of RYR-1 gene fragment

Extracted genomic DNA was identified using gel electrophoresis (Figure 1). The size of PCR product of the RYR-1 gene fragment is 656 bp, from nucleotide 18129 of intron 16 to nucleotide 18784 of intron 17 of the RYR-1 pig gene (Figure 2).

Table 1. The cutoff points of Hin6I on RYR-1 gene fragment

Allele	Cutting point	Base	Size (bp)
N	+1843	C	489/167
n		-	T 656

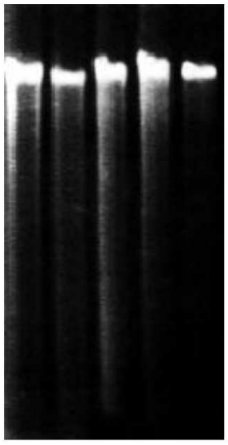

Figure 1. Total DNA extracted from pig ear tissue

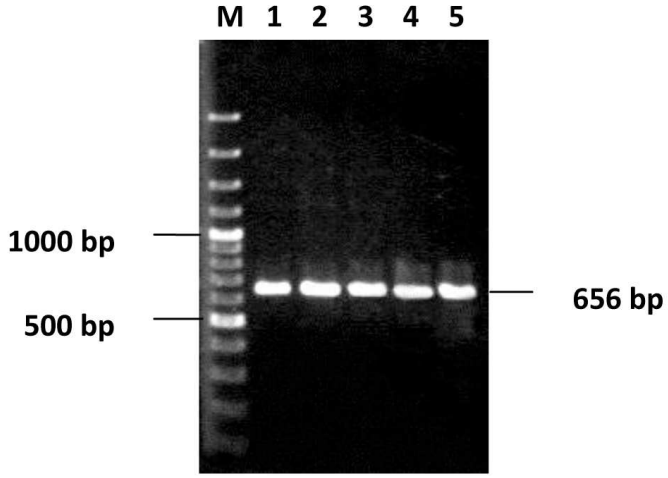

Figure 2. The PCR product of RYR-1 gene fragmentation
(M: DNA marker 100 bp; 1-5: PCR products have molecular size of 656 bp)

The single base-pair mutation of RYR-1 (C → T) is at the +1843 position of this gene. This mutation is located at restriction site of the Hin6I enzyme. The wild type allele (N) of the RYR-1 locus was cut by Hin6I to get 2 fragments of DNA (with molecular size 489 bp and 167 bp). If there is a mutation at the restriction site of Hin6I in the RYR-1 gene, the DNA fragment will not be cut and it produces a fragment of the size 656 bp, called

mutation allele *n* (Table 1). Therefore, the RYR-1 genotype of each individual pig can be determined by the number of bands observed after fragmentation using PCR production (Figure 3).

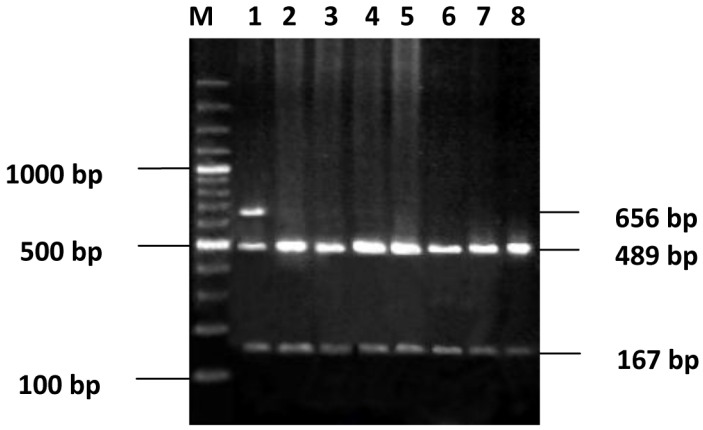

Figure 3. Fragments resulting from RYR-1 gene fragmentation using the Hin6I enzyme

(M: DNA marker 100 bp, 1: Nn genotype, 2-8: NN genotype)

2.2.2. Frequencies of RYR-1 genotypes

Of the collected samples, only 2 were of genotype *NN* and *Nn* with no *nn* genotype observed. There was only *NN* genotype observed among the sample Mong Cai pigs. One of the 2 Yorkshire pigs had the *NN* genotype and the other one had the *Nn* genotype.

Table 2. Frequencies of RYR-1 genotypes in groups of pig samples

Genotype	Mong Cai (MC) F_0		Yorkshire (YS) F_0		YS x MC F1	
	n	***%***	***n***	***%***	***N***	***%***
NN	13	100	1	100	52	100
Nn	-	-	-	-	-	-
Nn	-	-	-	-	-	-
NN	6	100	-	-	7	58.4
Nn	-	-	1	100	5	41.6
Nn	-	-	-	-	-	-
Total	19		2		64	

Table 2 shows that *NN* genotype frequency was high in local pigs (100% of the Mong Cai pigs) while only one Yorkshire pig had the *NN* genotype and the other Yorkshire had *Nn*. These results suggest that frequencies of allele *n* of imported pigs may be higher than in local pigs and therefore one might expect that the danger of PSS disease in local

pigs would be low. This is consistent with results reported by Nguyen Thu Thuy [8]. In that research, only 1.18% Mong Cai pigs had *Nn* genotype and 4 other local pigs (Co, Meo, Muong Khuong and Tap Na) all were of *NN* genotype. On the other hand, the frequencies of *NN, Nn* and *nn* genotypes of Landrace and Yorkshire pigs were 75%, 21.43%, 3.57% and 94.44%, 5.56% and 0%, respectively. These results indicate that local pigs have high pork quality, but low yield and low muscular weight while some imported pigs have high yield and high muscular weight, but lower pork quality.

2.2.3. Master Mix kit for determining RYR-1 genotype in pigs

** Design:*

The Master Mix kit consists of 2 components:

- The PCR Master Mix kit (*Taq* DNA polymerase 5U/μl, *Taq* Buffer with 10x KCl, 25 mM $MgCl_2$, 2.5mM dNTPs, 10 pM RYR-1 F and 10 pM RYR-1 R).

- Restriction enzyme kit (Buffer solution 10x and Enzyme Hin6I 10U/μl).

** Test:*

The results of testing the kit as below:

- Stability: The kit was stored at -20^{0}C for 60 days and it was used to determine the genotype of 35 samples, 3 times repeated. The results showed that 98.09 $\pm$ 3.30% of reactions were successful.

Figure 4. The Master Mix kit for determining RYR-1 genotype in pigs

Table 3. Test results demonstrating the stability of the Master Mix kit

Experiments	**Total number of samples**	**Percentage of successful reactions**	
		Number of samples	***Percentage (%)***
1st time	35	35	100
2nd time	35	33	94.29
3rd time	35	35	100
Mean $\pm$ SD	35	34.33 $\pm$ 1.15	98.09 $\pm$ 3.30

- Sensitivity: The kit was used to amplify the RYR-1 gene fragment of the samples at various DNA concentrations. As the results show, the amount of PCR product is reduced while the concentration of DNA sample decreases. The kit can amplify samples that have a minimum DNA concentration of 0.0032 μg/μL.

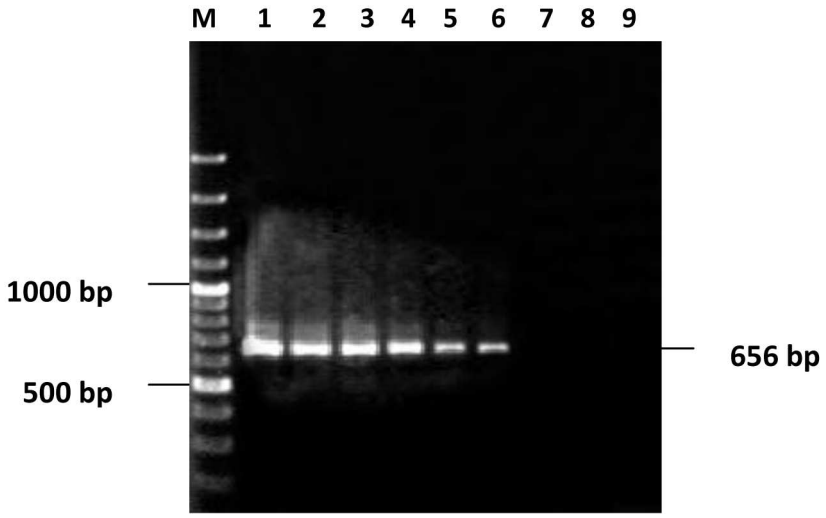

Figure 5. Test result demonstrating the sensitivity of the PCR Master Mix kit M-Marker ADN 100bp

Sample 1: 4 μg; sample 2: 1.6 μg; sample 3: 0.8 μg; sample 4: 0.4 μg; sample 5: 0.27 μg; sample 6: 0.2 μg; sample 7: 0.16 μg; sample 8: 0.08 μg and sample 9: 0 (control)

3. Conclusion

After analyzing the genetic diversity of the RYR-1 gene, it was found that Mong Cai pigs had only homozygote *NN* while imported Yorkshire pigs had both *NN* and *Nn* genotypes. Among their F1 generation, five out of 64 pigs were *Nn* genotype and the others were *NN* genotype. The Master Mix kit for determining the RYR-1 genotype in pigs was tested to show stability (98.09 $\pm$ 3.30% samples were amplified successfully) and sensitivity (0.0032 μg/μL is the minimum DNA concentration that the kit can measure).

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