International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:10, No:2, 2016

Association between Single Nucleotide Polymorphism of Calpain1 Gene and Meat Tenderness Traits in Different Genotypes of Chicken: Malaysian Native and Commercial Broiler Line

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***Abstract*—**Meat Tenderness is one of the most important factors affecting consumers' assessment of meat quality. Variation in meat tenderness is genetically controlled and varies among breeds, and it is also influenced by environmental factors that can affect its creation during rigor mortis and postmortem. The final postmortem meat tenderization relies on the extent of proteolysis of myofibrillar proteins caused by the endogenous activity of the proteolytic calpain system. This calpain system includes different calcium-dependent cysteine proteases, and an inhibitor, calpastatin. It is widely accepted that in farm animals including chickens, the μ-calpain gene (CAPN1) is a physiological candidate gene for meat tenderness. This study aimed to identify the association of single nucleotide polymorphism (SNP) markers in the CAPN1 gene with the tenderness of chicken breast meat from two Malaysian native and commercial broiler breed crosses. Ten, five months old native chickens and ten, 42 days commercial broilers were collected from the local market and breast muscles were removed two hours after slaughter, packed separately in plastic bags and kept at -20ºC for 24 h. The tenderness phenotype for all chickens' breast meats was determined by Warner-Bratzler Shear Force (WBSF). Thawing and cooking losses were also measured in the same breast samples before using in WBSF determination. Polymerase chain reaction (PCR) was used to identify the previously reported C7198A and G9950A SNPs in the CAPN1 gene and assess their associations with meat tenderness in the two breeds. The broiler breast meat showed lower shear force values and lower thawing loss rates than the native chickens ($p<0.05$), whereas there were similar in the rates of cooking loss. The study confirms some previous results that the markers CAPN1 C7198A and G9950A were not significantly associated with the variation in meat tenderness in chickens. Therefore, further study is needed to confirm the functional molecular mechanism of these SNPs and evaluate their associations in different chicken populations.

***Keywords*—**CAPNl, chicken, meat tenderness, meat quality, SNPs.

I. Introduction

MEAT quality is an important aspect of the meat industry because of consumers' acceptability [1]. Consumers' acceptability of meat products is dependent on the tenderness of such product and tenderness is known to vary in meat product. However, tenderness is a complex attribute in breeding programs which make in difficult for breeders to adequately select for improved tenderness. Therefore, molecular technology approach has become a powerful method for identifying animals with particular genetic traits associated with the desired tenderness and the selection process can be done on young animals even before birth [2].

Calpains have been reported to be involved in muscle growth and development. They are also regarded as proenzymes that are regulated by Ca^{2+} binding and autoproteolytic modification [3]. Four calpain genes (i.e., μ-calpain gene, CAPN1; m-calpain gene, CAPN2; p94 gene, CAPN3; and μ/m-calpain, CAPN1.5) are reported to be generally expressed in chickens [4]. Among the calpain family members, CAPN1 degrades myofibrillar proteins under postmortem conditions and appears to be the primary enzyme in the postmortem tenderization process [5]. Regulation of CAPN1 activity has been correlated with variation in meat tenderness; thus, CAPN1 is a good candidate gene for tenderness [6].

Polymorphisms within the CAPN1 gene have been documented in association studies for meat quality traits, particularly for tenderness and marbling [7]–[12]. In broiler chickens, 4 polymorphisms, 3 synonymous SNPs (i.e., C2546T, G3535A, and C7198A), and one SNP within the 3'-UTR (G9950A) of the CAPN1 gene have been reported to have significant effects on meat tenderness [9]–[11]. However, there are no available data on the evaluation of the association between these SNPs in Malaysian native chickens. The Malaysian native chicken is a slow growing breed which is an important indigenous breed distributed in Peninsular Malaysia and some parts of Indonesia. Therefore, the objective of this present study was to identify 2 reported polymorphisms in CAPN1 gene and assess the relationships between the CAPN1 polymorphisms and meat quality variation between commercial broiler and native crossbred Malaysian chickens.

II. Material and Methods

A. Experimental Animals

In this study, two meat-type quality chicken populations were used which included native crossbred chickens known in Malaysia as village chicken or "Ayam Kampong", and a selection of broiler commercial chickens that are bred and

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raised specifically for meat production. Ten chickens of similar weight and age were obtained from each breed at a local market in Semarak, Nilai, Negeri Sembilan. The birds were slaughtered, weighed and packed in Styrofoam box filled with ice before being transported to the laboratory of Universiti Sains Islam Malaysia for further analysis.

B. Phenotypic Qualities

The carcasses were deboned and the breast meat was halved into two for the determination of meat quality measurements such as thaw loss, cooking loss and shear force. The thaw loss and cooking loss measurements were determined as described by [13]. The measurement of shear force was carried out using the method described as in [14]. Rectangular blocks of 10 by 10 mm cross sectional area with a height of 25 mm were cut from the cooked fillets. Shear force was determined by using a Stable micro system TA.XT plus texture analyser equipped with a Warner Bratzler shear blade which cut the meat samples perpendicular to the fibre direction.

C. DNA Extraction and Genotyping

DNA was extracted from breast meat tissue using InnuPREP DNA Mini Kit (AJ Innuscreen GmbH, Berlin, Germany). The routine thermal cycler (Eppendorf) PCR method was used to perform the genotyping. The primers used in this study were based on previous studies [2], [10]. Two pairs of specific primers for CAPN1 (C7198A, and G9950A) were designed according to the genomic sequence of the CAPN1 gene in the Gen-Bank database (accession No. NC_006090.1; Table I).

PCR was done using My Taq™ Red mix from Bioline in a system with a total reaction volume of 50 µl. The PCR conditions were as follows; 94ºC for 4 min, 35 cycles at 94ºC for 3 s, 53ºC for 1 min, 72ºC for 1 min for extension, and a final extension 72ºC for 7 min. The 50 µl reaction mixture contained 1 µl of primer (20 µM each), 25 µl my taq red mix, 2x, 100ng/25µl reaction of DNA template (1µl) and the final volume was made up to a total 50 µl per reaction (by adding remaining nuclease free water).

PCR amplification was detected by 2% agarose gel and the gels were electrophoresed at 70 volts for 60 min. After this, the gels were stained with ethidium bromide solution (0.5 µg/ml) for 30 min at room temperature. The bands were viewed in a dark room using dark reader transilluminator (Clare Chemical Research).

TABLE I
SEQUENCES FOR EACH PAIR OF PRIMERS

Primers	Sequence of the primer (5'-3')	Length of the product
7198C/A-forward	GGTTCAGCAGGTTGTGCTTT	216
7198C/A-reverse	AGAGAGCCGAGCCCTAGTTC	
9950G/A-forward	TCA GGA CAC TGG TGT TCA ATA	212
9950G/A-reverse	GGA AAG GGT GTA GTG GTA C	

D. Statistical Analysis

All data obtained were analyzed using one-way analysis of variance. Association analyses of single polymorphisms with meat quality were determined using a general linear model procedure in the Minitab 17 software. The level of significance was set at $P < 0.05$.

III. RESULTS

A. Phenotypic Analysis and Meat Quality

The meat quality traits of both broiler and native Malaysian chickens are presented in Table II. The slaughter weight of the Malaysian native breed chicken was significantly ($P<0.05$) less than that of broiler commercial chickens. This is probably due to the fact that native Malaysian chickens are slow growing breed which weigh between 1.0-1.6 kg at 5 months. However, the broiler chickens are referred to as the meat type chicken because of their excellent feed conversion ratio and reaching market weight (2 kg) within 6 weeks [15]. The water holding capacity of the two chicken types were measured by the percentage thaw and cook losses. The percentage of thaw loss of native chicken was significantly ($P<0.05$) higher than those recorded for broiler chicken in term of breast meat. The result is similar to that obtained by [16] in which the crossbred native Thai chicken had a higher thaw loss percentage than other commercial types and attributed the difference to the different genotypes that hold the water molecule in meat. Although the percentage cook loss was not significantly ($P>0.05$) in both chicken types, however, the values recorded for the native chicken was higher compared to the cook loss recorded for the broiler chicken.

It has been reported that the slow-growing chicken meat tends to have a longer time of rigor inset with lower ultimate pH compared to broiler meat resulting in lower water holding capacity [17], [18], [25]. In addition, [19] indicated that percentage drip loss, thaw loss and cooking loss were reported to be significantly affected by genotype in slow-growing broilers. This result is in agreement with [20] that reported a higher drip loss and thaw loss in breast from slow-growing broilers when compared with the fast-growing broilers.

The result from the shear force showed that native chicken had significantly ($P<0.05$) higher shear force value compared to the broiler chicken. This result was similar to the findings of [16], [21]–[23]. They reported that toughness associated with native chickens might be related to slaughter age and production systems.

TABLE II
MEAT QUALITY CHARACTERISTICS OF BROILER AND NATIVE MALAYSIAN CHICKENS

Traits	Broiler chicken	Native chicken
Slaughter weight (kg)	1.98±0.11	1.41±0.21
% Thaw loss	2.50±1.08	5.93±2.05
% Cook loss	21.70±1.49	22.33±1.51
Shear force (kg)	1.98±0.57	3.80±0.41

Means ± standard deviation (n=10)

B. Genotype Study

The PCR method was used for the detection of nucleotide sequence polymorphism in the markers C7198A and G9950A in the CAPNl gene. The target fragments of the gene were

amplified and denatured, and was found using agarose gel electrophoresis as shown in Fig. 1.

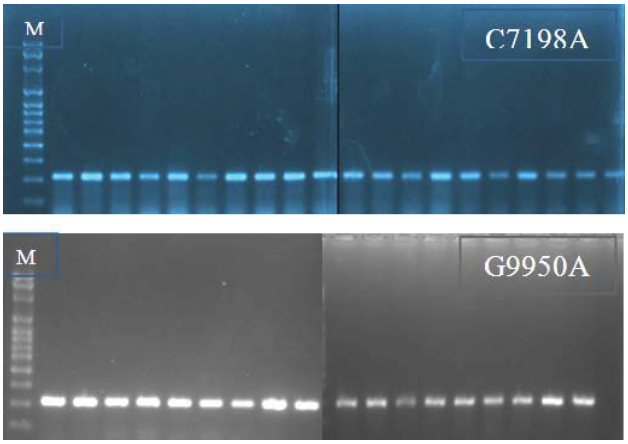

Fig. 1 Bands produced for all samples of both broiler and native chickens. M for DNA marker (100 bp). The bands sizes for loci C7198A were 216bp, and for loci G9950A were 212bp

C. Gene and Genotype Frequencies

The CAPNl polymorphisms detected by the PCR amplification were also confirmed by multiple sequencing of both populations in the markers C7198A, and G9950A SNPs. A part of the results of nucleotide sequence of SNPs in the CAPN1 gene was shown in Fig. 2. The comparisons of allele frequencies between the two loci SNPs are summarized in Table III. The marker C7198A was polymorphic in both breeds but the allele C was more frequent than the allele A in the native chickens. For CAPN1 G9950A, the allele G was dominant in both genotypes whereas the frequency of allele A was observed zero in the commercial broilers and was much less frequent than the allele G in the native chickens. There were no significant differences in allelic frequencies between native and commercial chicken samples ($P > 0.05$).

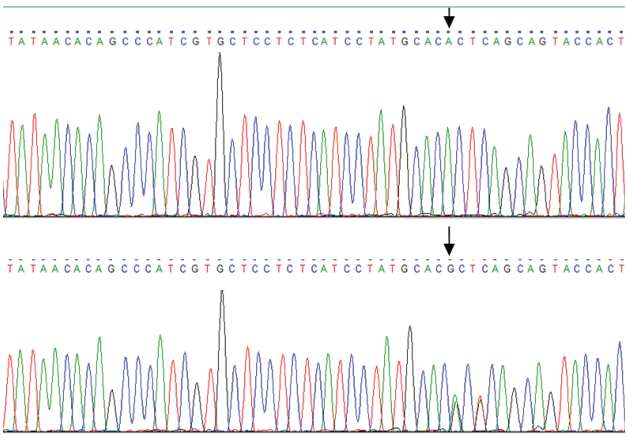

Fig. 2 The mutation in nucleotide sequence of CAPN1 G9950A SNP

TABLE III
ALLELIC FREQUENCIES OF THE 2 SITES IN THE CHICKEN POPULATIONS

Breed	N	C7198A		G9950A	
		C %	A %	G %	A %
Broiler commercial	10	0.50	0.50	1.00	0.00
Native chicken	10	0.70	0.30	0.80	0.20
X^2		0.833		2.222	
p-value*		0.361		0.136	

*Means the probability.

D. Polymorphism Association Analysis

One of the objectives of this study was to evaluate the association of SNPs with meat quality traits and the effect of genotypic differences between breeds. Previous research has reported a significant association between G9950A single polymorphism in the CAPN1 gene and the muscle fiber and the carcass traits [9], [10]. However, according to the results of multiple sequence comparison (Table III) the polymorphism was not more likely to be significantly associated with the differences in meat quality traits in the Malaysian crossbred lines. The results confirm the previous research that showed no significant association between these SNPs and breeds and their meat quality traits [2]. Additionally, the allele frequencies obtained from the Malaysian chicken populations differ significantly from those documented in other populations studied to date [2], [10]. Moreover, Allele G in the SNP 9950 CAPN1 was dominant allele and became fixed in the commercial chicken, thus the allele A was extinguished, thus the marker was not fitted with Hardy-Weinberg equilibrium. Therefore, a much larger sample size is needed to see any homozygous mutant for the SNPs.

IV. DISCUSSION

This study was to test some of the previously reported associations between the CAPN1 SNPs and meat quality traits and the effect of genotypic differences between chicken breeds. The results are the first to document the SNPs of the CAPN1 gene in Malaysian native chickens and commercial broilers.

The regions C7198A, and G9950A SNPs in the CAPN1 gene detected by the PCR amplification were confirmed by multiple sequencing and can be considered as selectable markers for CAPN1 gene. However, the allele frequencies of the SNP markers appeared to differ significantly from those documented in other populations studied to date [2], [9], [10]. According to our genotyping and sequencing results the marker C7198A SNP was polymorphic in both breeds but the allele C was more frequent than the allele A in the native chickens. For CAPN1 G9950A SNP, the allele G became fixed in the commercial samples while the allele A was lost in the commercial broilers and was much less frequent than the allele G in the native chickens.

As with genetic association, the polymorphisms C7198A, and G9950A in the CAPN1 gene were not significantly associated with the differences in meat quality traits between Malaysian chicken populations ($P > 0.05$). The results confirm recent work that found no significant associations between

these SNPs and breeds and their meat quality traits [2]. Additionally, these findings are in disagreement with the documented specific association between G9950A SNP and meat quality traits in Chinese chicken populations [10].

Based on the results obtained in this study, the SNPs C7198A, and G9950A in the CAPN1 gene were unable to explain the variations in meat tenderness and meat quality traits in selected Malaysian chicken populations. The reason for this contradictory may be due to a smaller population size. Furthermore, the individuals of the native chickens were randomly selected from the local market, and that could reduce the chance to differentiate their genotypic group [24]. Therefore, a pureline selection process with a sufficiently larger sample size is needed for further study on SNPs in the CAPN1 gene.

Previous research has also confirmed other factors that alter the phenotypic data, such as differences in age as well as gene-environmental factors [25]. The native Malaysian chickens are slow growing breed and so the toughness associated with native chickens' meat might be related to slaughter age and production systems. So it will be interesting to further investigate the differences in meat tenderness traits between the two groups, and how that can be attributed to different genotype-environmental factors.

V. Conclusion

The results obtained from this study have showed that the previously reported SNPs of CAPN1 gene were not significantly associated with variation in tenderness of broiler and Malaysian native chickens. Hence, the genetic markers need further studies to identify and confirm their correlations for other chicken populations.

Acknowledgment

The authors would like to sincerely thank the faculty of Science and technology, USIM for providing funding and necessary logistics for this research work.

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