CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical and Equipment

Chemicals and equipment used in this study was listed according to the methodology.

- WBSF T A. X T plus texture Analyzer
- DNA Isolation – InnuPREP DNA Mini Kit
- PCR – Master Mix, Nuclease free water, Primer, DNA Template
- Electrophoresis - Agarose Gel, 100bp ladder (Thermo Scientifc), Blue loading dye, 1X TAE Buffer
- Electrophoresis Power Supply – Biorad
- Nanophotometer – Nanophotometer P-class,AYKA LTD

3.1.2 Chicken Types and Sources

In this study, two meat-type quality chicken populations were used, including native crossbred chickens known in Malaysia as village chicken or “Ayam Kampong” and a selection of commercial broiler chickens “*Gallus gallus domesticus*” that are bred and raised specifically for meat production. Ten chickens from each population were randomly collected from the local market of Semarak, Nilai, and were weighted after 2-3 hours from slaughtering. The carcasses were separately placed and packed in plastic cooler filled with ice and transported to the laboratory of Universiti Sains Islam

Malaysia. Broiler chickens were 42 days of age and weight between 1.80 and 2.10 kg. The native chickens, which are known of slow-growing breed, were five months at slaughter and weight between 1.0 and 1.6 kg post-mortem.

3.1.3 Sample Collection

Twenty breast meat pieces were cut and removed as samples from carcasses. Each separated breast was divided into two halves (Figure 3.1), one half was prepared and packed in zipper plastic bags for meat quality measurements and the other half was packed and refrigerated for DNA extraction.

Figure 3.1: Preparation of Breast Meat Samples

3.2 Meat Quality Parameters Measurements

3.2.1 Thaw loss

The Twenty halves of breast meat from earlier step were prepared by removing skin and deboning. The samples were weighed at 5-7 h post-mortem and stored at -20ºC for 24 h refrigeration. The samples were taken out from refrigerator and kept at 4ºC overnight. The pieces samples were reweighed. Thaw loss was calculated as the difference in the weight of sample before freezing and after thawing.

$$\text{\% Thaw Loss} = \frac{\text{Sample weight prior freezing} - \text{Sample after thawing}}{\text{Sample Weight prior freezing}} \times 100$$

3.2.2 Cook Loss

Overnight refrigerated breast were individually packaged in plastic bags, sealed and cooked to an internal temperature of 75±1ºC by dipping a mercury thermometer (GH Zeal LTD-London-England) to monitor the temperature in an 80±0.5ºC water bath (WNB7, Memmert GmbH) for 25-35 min. Samples were immediately cooled in water for 20 minutes, and then weighed. Cooking loss was calculated as the percentage weight lost during cooking (Dadgar et al., 2010).

$$\text{\% Cook Loss} = \frac{\text{Sample weight after thawing} - \text{Sample Weight after Cooking}}{\text{Sample Weight after thawing}} \times 100$$

3.2.3 Warner- Bratzler Shear Force (WBSF) Test

The previously cooked samples were wrapped with aluminum foil after cooling and held at 4ºC overnight. On the next day, triplicate block samples from each cooked fillet were prepared for Warner-Bratzler shear force test (Fernandez et al., 2001). The blocks are 10 ×10 mm cross section with a height of 25 mm parallel to the muscle fiber direction. 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 fiber direction as illustrated in Figure 3.2.

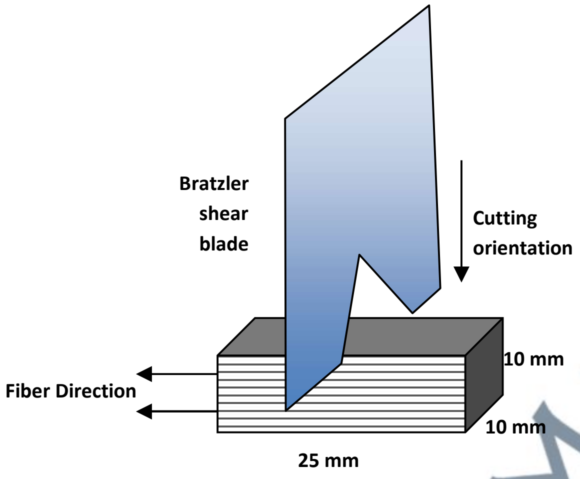

Figure 3.2: Warner- Bratzler Shear Force (WBSF) Test

3.3 Genotype Study

3.3.1 Extraction of total DNA from tissue

InnuPREP DNA Mini Kit was used to extract the total DNA from the other half of each chicken tissue breast that was previously prepared. The sample with a weight of 0.050 g was extracted 1cm depth from the upper side of the breast and then inserted into 1.5 ml reaction tube. The 400 µl of Lyses Solution TLS and 25 µl of the proteinase K were added to the tube. The tube was mixed using a vortex for 5 seconds. The tube that contains the mixture was incubated at 50ºC for 3-4 hours until the sample is completely lyses. Water bath was used for incubation. To increase the lyses efficiency, the sample was vortexed each 10 minutes during the incubation process. Next, the 1.5 ml tube was put into micro centrifuge machine at 12.000 rpm for 1 minute to spin down unlysed material. The supernatant was transferred into another 1.5 ml tube. Then 400 µl Binding Solution TBS was added to the lyses sample and vortexes for 15 seconds.

A spin filter tube located in a 2.0 ml receiver tube was prepared to pore the sample in it. The cap was closed and centrifuged at 12.000 for 2 minutes. Then the receiver tube with the filtrate was discarded. The spin filter tube was placed into a new 2.0 ml receiver tube. 500 µl of Washing Solution HS was added to the spin filter. Next, the spin filter was centrifuged at 12.000 rpm for 1 minute. Then the receiver tube was discarded with the filtrate, and the spin filter was placed into a new 2.0 ml receiver tube. After been placed in new receiver tube, 750 µl of washing solution MS was added to the spin filter and then centrifuged at 12.000 rpm for 1 minute. Then the spin filter tube was spin in micro centrifuge at maximum speed for 2 minute after placed into new 2.0 ml receiver tube, to remove all traces of ethanol.

The spin filter column was transferred into new 1.5 ml reaction tube to receive the desired solution. Then 200 µl of elution buffer was added into the spin filter and was incubated at room temperature for 1 minute. Then the tube contain sample was spin for 1 minute at 8000 rpm. Then the nanophotometer was used to determine the concentration of the extracted DNA.

3.3.2 Selection of candidate genes

The CAPN1 gene was selected based on previous functional evidence indicating that this gene is associated with meat quality traits.

3.3.3 PCR Primer Design

The appropriate primers sequences required for the amplification of CAPN1 gene are identified based on Zhang et al. (2007a, b), Zhang et al. (2008) and Shun et al. (2015). The primers were designed to amplify the target fragments in CAPN1 gene using OLIGO 6.0 according to chicken genomic sequence in the GenBank database

(Accession Number: NC_006090.1). Five primers were designed using the Primer-Blast tool of National Center of Biotechnology Institute (NCBI) website (Accession Number: NM_001044672.1) to be suited for the target regions. Primers synthesis was completed by Integrated DNA Technology Corporation, Singapore. The Primers pairs that designed to detect the polymorphic regions are presented in Table 3.1.

Table 3.1: Possible primers used for PCR reaction.

Primer	Forward (5'-3')	Reverse (5'-3')	GC%	Product length (bp)
Pair 1 (3535G/A)	TCACCTGAGGTTTGCATGTT	AGCCATCAAATGCAGGAAGT		190
Pair 2 (7198C/A)	GGTTCAGCAGGTTGTGCTTT	AGAGAGCCGAGCCCTAGTTC	55.00	216
Pair 3 (9950G/A)	TCA GGA CAC TGG TGT TCA ATA	GGA AAG GGT GTA GTG GTAC	55.00	212
Pair 4	GGAGGCGGTGACATTCAAGA	GGGTTGATCCAAAACGTGGC	55.00	405
Pair 5	GGCTTTGCTGTCTACGAGGT	TTTCTTCGGTGTCGGCTCTC	55.00	247
Pair 6	GAGAGCCGACACCGAAGAAA	TGACCATACTGCGACACGAC	55.00	233
Pair 7	TGGAGACCATGTTCCGCTTC	ATCCCTGATTCGCTGCCATC	55.00	390
Pair 8	GCCTCTTCAGGGACGAAACCT	CTTCATAGCACCCGTTCACCT	57.14	450

3.3.4 Polymerase Chain Reaction (DNA Amplification)

Amplification of the DNA fragments was in a 50 µl of the total reaction volumes. The standard PCR reaction carried out in this experiment used the My Taq^{TM} Red mix from Bioline. The PCR reaction mixtures contained 1 µl of forward 5' and reverse 3' primer (20 µM each), 25 µl of my taq red mix, 2x contained all the reagents including [10X Buffer w/o $MgCl_2$, 50 mM $MgCl_2$, dNTP (10mM)], 1µl of DNA template and the final volume being made up to a total 50 µl per reaction (by adding remaining nuclease free water). The composition of each ingredient is shown in Table 3.2.

The reaction was placed in a routine thermal cycler (Eppendorf) that has been preheated to 95ºC. The reaction was incubated or through a pre-denaturation in a PCR thermocycler at 94ºC for 4 minutes to activate the Taq DNA polymerase enzyme before moving into amplification cycles. The amplification cycle contained three steps: denaturation of DNA strand at 94°C for 30 seconds, annealing of primers at 53ºC for 1 minute and elongation by polymerase enzyme at 72ºC for 1 minute. Process of denaturation, annealing, and extension were set to repeat for 35 cycles to increase the yields of PCR product, followed with the final extension of polymerase enzyme at 72°C for 7 minutes and, finally, cooling of the amplified product to 12°C at which temperature it was held indefinitely. The setting of the Thermal Cycler and time for each step are presented in Table 3.3.

Table 3.2: Composition of the ingredient for each PCR run

PCR Master Mix	**25 µl**
Forward primer (20 µM)	1 µl
Reverse primer (20 µM)	1 µl
DNA template	1 µl
Nuclease free water	23 µl
Total	50 µl

Table 3.3: Thermal cycling conditions for PCR amplification

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	94ºC	4 minutes	1 cycle
Denaturation	94ºC	30 seconds	
Annealing	53ºC	1 minute	
Extension	72ºC	1 minute	35 cycle
Final extension	72ºC	7 minutes	
Soak	12ºC		

3.3.5 Gradient PCR

The gradient PCR was used in this study to optimize the annealing temperatures for primer binding. Moreover, analysis on the effect of different annealing temperature on the amplification of specific and non-specific amplicon was also been done. PCR reaction was prepared as described in section 2.3.2. The gradient PCR thermocycler (eppendorf) was set to run with a different annealing temperature across the wells as shown in Table 3.4. The temperature for annealing in the gradient PCR thermocycler was increased up to 7°C and decreased to 1°C from its original setup temperature at 53°C, while the general cycling conditions were kept unchanged. The construction of 7 wells gradient block in a gradient PCR thermocycler is shown in Figure 3.3.

Table 3.4: The PCR reaction and gradient annealing temperature

Initial Denaturation		94°C	4 minutes	1 X
Denaturation		94°C	30 second	35X
Annealing	A	58.0°C	1 minutes	
	B	57.5°C		
	C	56.8°C		
	D	55.7°C		
	E	54.3°C		
	F	53.1°C		
	G	52.4°C		
	H	52.0°C		
Extension		72°C	1 minutes	
Final extension		72°C	7 minutes	

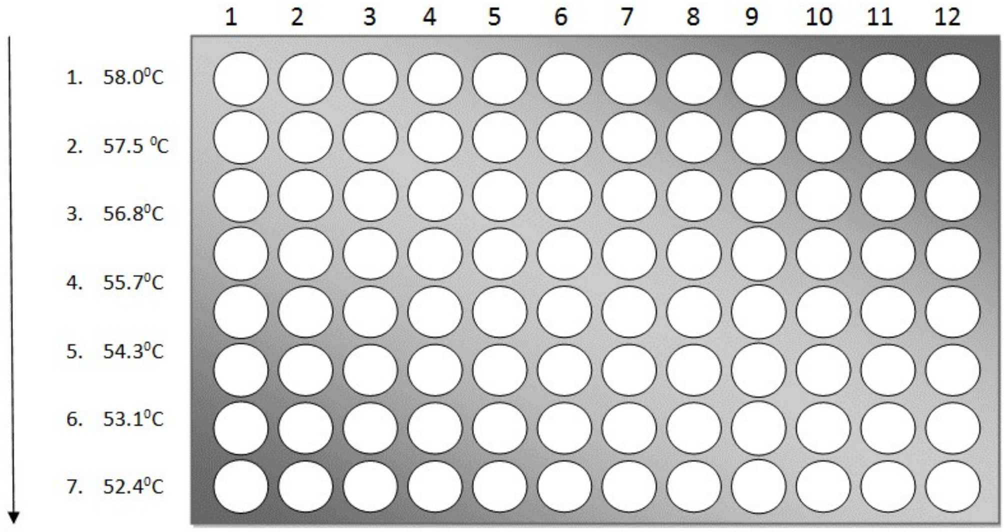

Figure 3.3: Construction of 7 wells gradient block in a gradient PCR thermo cycler.

Annealing temperatures for PCR reaction can be set up to vary across the block during PCR reaction. Shown in the image was gradient annealing temperature increase 7°C and decrease of 1°C from its original setup temperature at 53°C.

3.3.6 Agarose Gel Electrophoresis

Agarose powder of 1.2 g was weighed and added to 60mL of 1X TAE buffer (0.04 M Tris acetate 0.001 M EDTA, and pH 8.0) for preparing 2% (w/v) gel. The agarose/buffer mixture was then heated in microwave for 1.5 minute and was left to cool and to pour into a gel casting tray (Nyx Technik). A gel comb was placed onto the gel to create wells before it solidifies. The solidified gel was placed in the gel tank and the remaining diluted (1X TAE) buffer was used to fill in the tanks. In order to electrophorese DNA samples, 10 µl of DNA sample was being loaded into a well, 6 µl of HyperLadder^{TM}100bp Plus (Bioline) was used as a marker for DNA size (Figure 3.4). The loaded DNA samples were electrophoresed at 70 v and 400 A for 45 minutes.

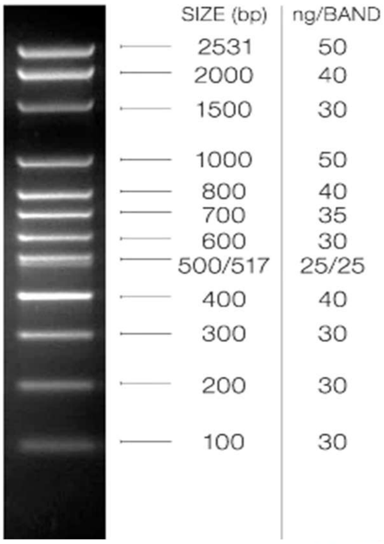

Figure 3.4: Hyper Ladder 100bp plus on a 2% agarose gel and visualized using ethidium bromide.

3.3.7 Visualizing DNA on Gels

The gel that has been electrophoresed was sequentially stained with ethidium bromide solution (0.5 µg/ml) for 30 minutes at room temperature, rinsed and destained with distilled water. Then it was viewed by using dark reader transilluminator (Clare Chemical Research) in a dark room.

3.3.8 DNA sequencing

The unpurified PCR products for all samples and the three primers pairs (G3535A, C7198A, and G9950A) were subjected to DNA sequencing to assist data analysis especially for the variations in the nucleotide sequences. The sequencing was carried out by First Base Laboratories Sdn. Bhd in Seri Kembangan, Selangor. The samples were sequenced using BigDye® Terminator v3.1 cycle sequencing kit.

3.4 Statistical Analysis

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