COMPARATIVE ANALYSIS OF *BoLA-DRB3* ALLELIC VARIATION TO DETERMINE GENETIC DIVERSITY AND DISEASE RESISTANCE IN KEDAH–KELANTAN (KK) AND MIYAZAKI WAGYU CATTLE

By

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2025

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COMPARATIVE ANALYSIS OF *BoLA-DRB3* ALLELIC VARIATION TO DETERMINE GENETIC DIVERSITY AND DISEASE RESISTANCE IN KEDAH–KELANTAN(KK) AND MIYAZAKI WAGYU CATTLE

ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement of the course DVT 55204 - Research Project.

This study aimed to establish and validate a Polymerase Chain Reaction (PCR) assay for the amplification of the *BoLA – DRB3* exon 2 region in Kedah – Kelantan (KK) and Miyazaki Wagyu cattle, providing a foundational step for comparing their immunogenetic diversity. Blood samples were collected from twenty clinically healthy animals (n = 10 per breed) in Kelantan, Malaysia. Genomic DNA was extracted and amplified using primers specific for *BoLA – DRB3* exon 2. Agarose gel electrophoresis confirmed successful amplification in 19 out of 20 samples, yielding a primary amplicon of approximately 300 base pairs (bp), which aligns with the expected fragment size range for this locus. One Miyazaki Wagyu sample showed no amplification, likely due to technical issues. The consistent amplification across both breeds validates the employed primers and PCR protocol. This successful genotyping establishes the essential molecular groundwork for subsequent, high – resolution analyses such as sequencing, which are required to identify specific alleles and investigate their potential association with disease resistance in Malaysia's tropical production environment.

Keywords: Malaysia, Kelantan, Miyazaki Wagyu, Kedah - Kelantan (KK) cattle, *BoLA- DRB3*.

ANALISIS PERBANDINGAN VARIASI ALEL *BoLA - DRB3* UNTUK MENENTUKAN KEPELBAGAIAN GENETIK DAN KETAHANAN PENYAKIT DALAM LEMBU KEDAH - KELANTAN (KK) DAN MIYAZAKI WAGYU

ABSTRAK

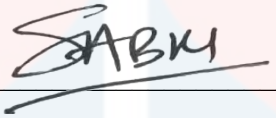
Abstrak daripada kertas penyelidikan yang dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan, sebagai keperluan sebahagian daripada kursus DVT 55204 - Projek Penyelidikan.

Kajian ini bertujuan untuk membangun dan mengesahkan ujian Tindak Balas Berantai Polimerase (PCR) untuk amplifikasi rantau ekson 2 *BoLA – DRB3* dalam lembu Kedah – Kelantan (KK) dan Miyazaki Wagyu, sebagai langkah asas untuk membandingkan kepelbagaian immunogenetik mereka. Sampel darah telah diambil daripada dua puluh ekor lembu yang sihat secara klinikal (n = 10 setiap baka) di Kelantan, Malaysia. DNA genom telah diekstrak dan diamplifikasi menggunakan primer khusus untuk ekson 2 *BoLA – DRB3*. Elektroforesis gel agarosa mengesahkan amplifikasi yang berkaya dalam 19 daripada 20 sampel, menghasilkan amplikon utama pada kira – kira 300 pasangan bes (bp), yang sejajar dengan julat saiz jangkaan untuk lokus ini. Satu sampel Miyazaki Wagyu tidakk menunjukkan amplifikasi, berkemungkinan disesbabkan oleh isu teknikal. Amplifikasi yang konsisten merentas kedua – dua baka ini mengesahkan primer dan protokol PCR yang digunakan. Penjenisan genotip yang berjaya ini mewujudkan asas molekul yang penting untuk analisis beresolusi tinggi seterusnya seperti penjujukan, yang diperlukan untuk mengenal pasti alel spesifik dan menyiasat perkaitannya yang berpotensi dengan ketahanan penyakit dalam persekitaran pengeluaran tropika Malaysia.

Kata kunci: Malaysia, Kelantan, Miyazaki Wagyu, lembu Kedah - Kelantan (KK), *BoLA – DRB3*.

CERTIFICATION

This is to certify that we have read this research paper entitled "**Comparative analysis of *BoLA - DRB3* allelic variation to determine genetic diversity and disease resistance in Kedah – Kelantan (KK) and Miyazaki Wagyu cattle**" by Siti Hajar Afifah Binti Hamrah, and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirements for the course DVT 55204 - Research Project.



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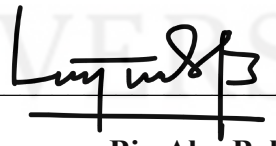

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Norlaila Binti Najib Paarthiban

My cats (Dahlia, Kasturi and Mister)

DVM 5 Class of 2021/2026

Myself

Thank You

DEDICATIONS

وَمَا تَوْفِيقِيٓ إِلَّا بِٱللَّهِ

My success is only by Allah (swt)
Surah Hud [11:88]

First and foremost, I would like to praise Allah the Almighty for making it all possible for me to complete my final year project, for showing me that there is a light to every tunnel. I would like to take this opportunity to acknowledge everyone who has helped me along the way.

Special thanks to the owner of Kelfarm Sdn. Bhd., Encik Paizol Akman Bin Awang for giving me the opportunity to do sampling and research on your farm. Without this opportunity, I will not be able to fulfill the needs for this project.

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Table 4.4.2.2 For the detection of specific blood pathogens using their respective primers, the expected fragment (band sizes) were as follows.

LIST OF ABBREVIATIONS

BoLA – DRB3	Bovine Leukocyte Antigen Complex
PCR	Polymerase Chain Reaction.
PCR - RFLP	Polymerase Chain Reaction and Restriction Fragment Length Polymorphism
BCS	Body Condition Score.
KK	Kedah - Kelantan.
MAS	Marker Assisted Selection
SNPs	Single Nucleotide Polymorphisms
BLV	Bovine Leukemia Virus

LIST OF SYMBOLS

%	Percentage
&	And
^{o}C	Celcius
ml	Millilitre
l	Litre
n	Nano
µ	Micro
bp	Base pair

CHAPTER 1

INTRODUCTION

1.1 Research background

The beef cattle industry in Malaysia consists of both indigenous and imported breeds, with increasing interest in genetic improvement to enhance productivity and disease resistance (Rahman et al., 2019). Kedah - Kelantan (KK) cattle, a local zebu - type breed, are well adapted to Malaysia's hot and humid tropical climate and demonstrate resilience against endemic diseases due to long - term natural selection (Jamaludin et al., 2021). In contrast, Miyazaki Wagyu was renowned for its superior marbling and excellent meat quality which shows limited adaptability to high temperatures and humid environments, making it more susceptible to heat stress and local pathogens (Sasaki & Nishimura, 2018).

One important genetic marker used to compare disease resistance potential in both breeds is *BoLA - DRB3*, which is also called as bovine leukocyte antigen complex is a highly polymorphic locus within bovine major histocompatibility complex (MHC) class II region (Lewin et al., 1999). Polymorphisms, particularly within exon 2, play a major role in antigen presentation and pathogen recognition, influencing overall immune competence (Behl et al., 2012; Takeshima & Aida, 2020).

This study aims to compare Miyazaki Wagyu breed in Kelantan and KK cattle using Polymerase Chain Reaction (PCR) fragment size analysis. Through PCR amplification and gel electrophoresis, researchers can verify successful amplification and infer polymorphisms based on fragment size differences. According to Ono et al., 2007, this approach does not provide nucleotide sequence information or detailed allele level characterization, which would require a sequencing-based method.

1.2 Research problem

BoLA - DRB3 is one of the most polymorphic immune related genes in cattle (Lewin et al., 1999), and its allelic variation has been linked to differential resistance or susceptibility to major infectious diseases such as bovine leukemia virus (BLV), mastitis, tick – borne diseases, bovine tuberculosis and foot and mouth disease (FMD). However, despite its proven relevance as a marker of immune competence, limited information exists on how *BoLA - DRB3* diversity differed between indigenous tropical breeds like KK cattle which known for strong environmental adaptability while imported breeds such as Miyazaki Wagyu, which are more susceptible to heat stress and local pathogens. This knowledge gap restricts the ability to evaluate disease resistance potential and limits the development of informed markers assisted breeding strategies. Therefore, further detection of the *BoLA - DRB3* gene between these two breeds is necessary to understand their genetic diversity and potential resilience against endemic diseases in Malaysia's tropical environment.

1.3 Research questions

1. Does the presence of the *BoLA - DRB3* gene can be detected in Kedah - Kelantan (KK) and Miyazaki Wagyu cattle by using PCR?
2. Is there any difference in detection frequency between these two breeds?

1.4 Research hypothesis

1. The traits of *BoLA - DRB3* gene can be detected in both cattle breeds.
2. KK cattle will have a higher PCR detection frequency of *BoLA - DRB3* gene compared to Miyazaki Wagyu cattle.

1.5 Research objectives

1. To detect the presence of the *BoLA - DRB3* gene in Kedah - Kelantan (KK) and Miyazaki Wagyu cattle by using PCR.

2. To compare the PCR detection frequency between these two breeds.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of the beef industry in Malaysia

According to Department of Veterinary Services (DVS) Malaysia (2023), the national beef industry is relatively small and dominated by smallholders and traditional farming systems, with indigenous breeds like Kedah - Kelantan (KK) playing a major role. Furthermore, Dzulkifli (2025) noted that the self-sufficiency level (SSL) for cattle and buffalo meat was only 15.9% in 2023, considerably lower than 35.5% predicted under the National Agrofood Policy 2.0 (DAN 2.0) and well short of its 50% target by this year, as highlighted in the 2024 Agriculture Census Interim Report (2024). As stated by Prof. Dr. Loh Teck Chwen, Dean of the Agricultural Faculty of University Putra Malaysia (UPM), this is caused by small - scale cattle operations and a lack of indigenous breeding stock. Malaysia currently faces a shortage of cattle breeders and parent stock, resulting in continued reliance on imported beef to meet domestic consumption needs. Imported breeds such as Wagyu, Brahman, and European crosses are used to improve carcass quality, but many perform poorly under Malaysia's hot and humid climate (Jamaludin et al., 2021).

Additionally, diseases related conditions including clinical and subclinical diseases further constrain the productivity, particularly blood-borne parasites. This may include theileriosis, trypanosomiasis and anaplasmosis which are highly prevalent in Malaysia due to the tropical environment and disproportionately affect exotic breeds (Chong et al., 2019; Shah et al., 2021; Kocan et al., 2015). Other common infectious diseases such as hemorrhagic septicemia, leptospirosis, bovine tuberculosis, FMD and lumpy skin disease (LSD) also contribute to economic losses (OIE, 2022). Combined with parasitism and biosecurity limitations, these factors highlight

the need for genetic approaches to improve resilience (Hogan & Walker, 2016). Therefore, evaluating immune – related genes such as *BoLA – DRB3* is vital to compare disease resistance between indigenous KK cattle and imported Miyazaki Wagyu, and to support future marker – assisted breeding programs (Miyasaka et al., 2018).

2.2 Importance of *BoLA - DRB3* gene in cattle

The *BoLA - DRB3* gene is one of the most polymorphic loci within the bovine major histocompatibility complex (MHC) and plays an important role in antigen presentation and activation of adaptive immunity in cattle (Aida et al., 2015). Its extensive allelic diversity influences how effectively cattle recognize and respond to pathogens, making certain alleles associated with disease resistance or susceptibility to conditions such as bovine leukemia virus (BLV), mastitis, theileriosis, trypanosomiasis, bovine tuberculosis, and foot and mouth disease (FMD) (Miyasaka et al., 2018; Takeshima & Aida, 2006). Because of these strong associations, *BoLA - DRB3* serves as a valuable genetic marker for immune competence, allowing researchers and breeders to assess disease resilience, identify superior animals and support marker assisted selection (MAS) to improve herd health and productivity (Takeshima et al., 2014).

2.3 Genetic predisposition of *BoLA - DRB3* gene

Wagyu cattle, particularly the Japanese Black strain, are intensively selected for superior meat quality rather than immune traits, resulting in reduced genetic diversity in immune related genes such as *BoLA - DRB3* (Miyasaka et al., 2018). Several studies reported that Wagyu cattle tend to carry *BoLA - DRB3* alleles associated with higher disease susceptibility and they often lack protective alleles such as *DRB30902,* which has been linked to resistance against mastitis and

bovine leukemia virus (BLV) (Takeshima & Aida, 2006; Miyasaka et al., 2018). This reduced allelic diversity is likely a consequence of intensive selection pressure and inbreeding for carcass traits, making Wagyu more vulnerable to infection under tropical conditions. However, this Miyazaki Wagyu was reared intensively in Thailand before being imported into Malaysia, which considers them to have exposure to the environmental conditions of Thailand, similar to Malaysia's tropical climate.

In contrast, the indigenous KK cattle, a hardy zebu type breed native to Malaysia, exhibit greater polymorphism in the *BoLA - DRB3* gene, reflecting long term natural adaptation to tropical pathogens (Rahman et al., 2020). Protective alleles such as *DRB30202*, *DRB31101* and *DRB32703* have been associated with resistance to BLV, mastitis and gastrointestinal parasites in tropical cattle populations (Sharif et al., 2015; Takeshima et al., 2014). The higher heterozygosity observed in KK cattle suggests a more diverse and responsive immune system, supporting their resilience in hot, humid and disease prone environments. These immunogenetic polymorphisms may underlie the breed's superior tolerance to endemic challenges such as tick infestation, helminthiasis, and blood borne parasitic infections.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Animal ethics statement

Ethical approvals for using animals in the current study was obtained from the Animal Ethics Committee, Faculty of Veterinary Medicine of University Malaysia Kelantan under the approval code of UMK/FPV/ACUE/FYP/016/2025.

3.2 Study area and target population

The study was carried out in two farms located in Kelantan, Malaysia. The study on Miyazaki Wagyu cattle was conducted at a designated farm located in Kota Seribong, which is a sub-district within the Limbat area of Kota Bharu District, Kelantan, Malaysia. This region is a part of the state's main development area, typically characterized by a mix of residential, commercial and agricultural activity. The study site falls under tropical rainforest climate prevalent in Kelantan, characterized by high temperatures, high humidity, and significant rainfall, particularly during the monsoon season. The study was conducted at the Kelfarm Sdn. Bhd which is a private commercial farm specializing in the intensive rearing of premium beef breeds. The facility is equipped with climate - controlled barns.

The study on Kedah - Kelantan (KK) cattle was carried out in Teaching Farm in Universiti of Malaysia Kelantan (UMK) Bachok, Kelantan, Malaysia. Bachok district is located in the northeastern part of Kelantan, bordered by the Kota Bharu District to the north and west. This region is known for its coastal and low - lying terrain, with common soil types including sandy, peat and clay soils. The climate is a hot and humid tropical climate, which the indigenous KK

cattle are uniquely adapted to. The cattle typically utilize the extensive system, which reflects the common management practice for this indigenous breed in the area.

3.3 Acquiring consent and subject medical history

Consent was given both verbally and written by the owner through ***Whatsapp*.** History was taken verbally and recorded on paper.

3.4 Sample collection

A general physical examination (PE) have been performed on each recruited animal, Miyazaki Wagyu cattle (n = 10) and Kedah - Kelantan (KK) cattle (n = 10) to verify clinical health and rule out any indications of systemic disease. This involves evaluating the body's temperature, mucous membranes, respiratory and cardiac rates, and overall health. Sterile 18-gauge needles and vacutainer devices were used to aseptically draw blood samples from the jugular vein or coccygeal vein. Approximately 10 mL of whole blood was drawn from each animal and immediately transferred into ethylenediaminetetraacetic acid (EDTA) tubes to prevent coagulation. All samples were clearly labeled and stored at 4°C before being transported to the UMK Zoonotic Laboratory for genomic analysis (Figure 3.4.1).

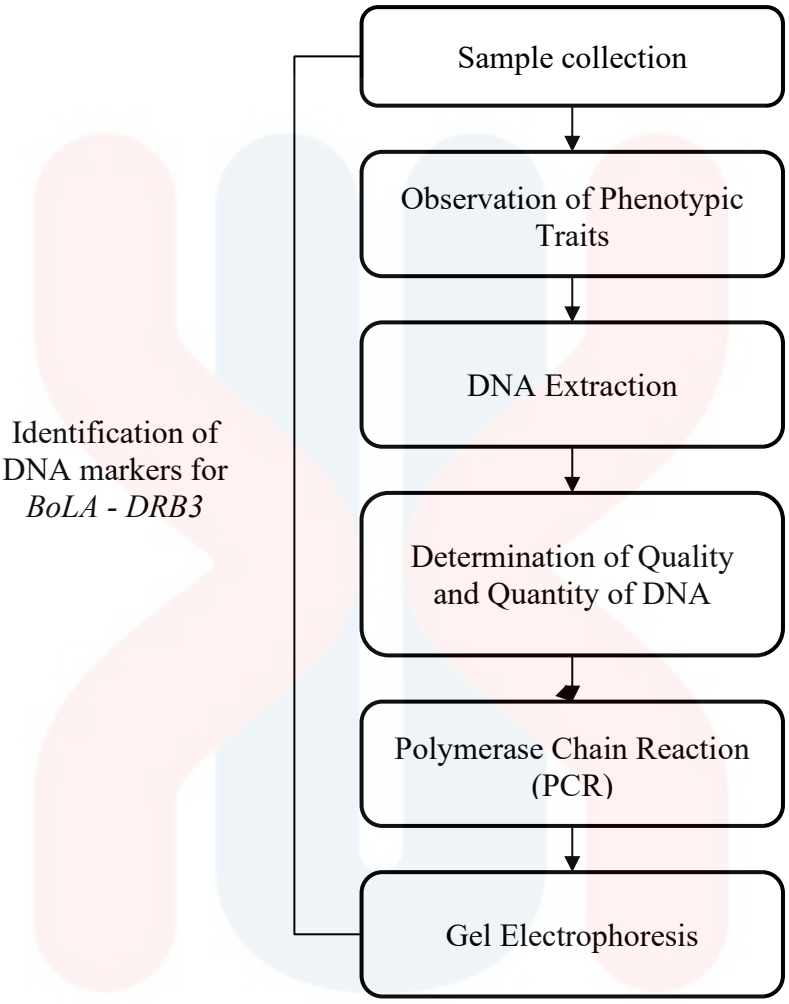

Figure 3.4.1: Schematic diagram of the procedure for identification of DNA markers for *BoLA - DRB3*.

3.5 Observation of phenotypic traits

Phenotypic observations were conducted on KK cattle (n = 10) and Miyazaki Wagyu (n = 10). All animals were clinically healthy and of comparable age. Observations were carried out under different management and environmental conditions to minimize external influences on phenotypic expression.

Phenotypic characterization was performed using direct visual observation and standard morphometric measurements, following guidelines for livestock breed characterization recommended by the Food and Agriculture Organization (FAO) (FAO, 2012).

It consists of qualitative and quantitative traits. For qualitative traits, it was assessed through visual observation and recorded based on predefined descriptors as described by FAO (2012) and Yakubu et al. (2010). The traits evaluated were coat color and coat pattern, skin pigmentation, horn presence and horn shape, ear orientation, hump development, dewlap size, and general body conformation. However, for quantitative traits, measurements are taken using a tape and a measuring stick on animals standing on a flat surface, following standard procedures described in previous cattle phenotypic studies (FAO, 2012; Yakubu et al., 2011). The measurements recorded included body length, height at withers, heart girth, and hip width. All measurements should be recorded in centimeters (cm). Unfortunately, we are only able to record the general observation, qualitative traits, but not quantitative traits.

3.6 DNA extraction

Total genomic DNA was extracted from the collected blood samples using the DNA Isolation Kit Blood, following the manufacturer's protocol. DNA extraction was performed immediately after sampling. For each sample, 300µl was transferred into a 1.5ml microcentrifuge tube. Firstly, the sample was prepared by adding 3X the sample volume of RBC Lysis Buffer then mixing by inversion. Do not vortex. Incubate the tube for 10 minutes at room temperature. Centrifuge for 5 minutes at 3,000 x g then remove the supernatant completely. RBC Lysis Buffer (100 µl) was added to resuspend the leukocyte pellet, after which cell lysis was initiated. GB Buffer (200 µl)

was then added to the sample, and the 1.5 ml microcentrifuge tube was shaken vigorously. The mixture was incubated at 60°C for at least 10 minutes until the lysate became clear. During incubation, the tube was inverted every 3 minutes. During this incubation period, the required Elution Buffer (200µl per sample) was preheated to 60°C in preparation for the DNA elution step. For DNA binding, 200 µl of absolute ethanol was added to lysate and immediately mixed by vigorous shaking for 10 seconds. A GD Column was placed into a 2 ml collection tube, and the entire mixture, including any precipitate, was transferred to the column. The column was centrifuged at 14,000 to 16,000 x g for 5 minutes. The collection tube was discarded, and the GD Column was placed into a new 2 ml collection tube. During the washing steps 400 µl of W1 Buffer was added to the GD Column, followed by centrifugation at 14,000 to 16,000 x g for 30 to 60seconds. The flow – through was discarded and the column was returned to the collection tube. Next, 600 µl of Wash Buffer (with ethanol added) was applied to the column and centrifuged at 14,000 to 16,000 x g for 30 to 60 seconds, after which the flow – through was discarded. The column was centrifuged again at 14,000 to 16,000 x g for 3 minutes to completely dry the column matrix. For DNA elution, the dried GD Column was transferred to a clean 1.5ml microcentrifuge tube. 100 µl of preheated Elution Buffer, Tris – HCl and EDTA (TE), or nuclease – free water was added to the center of the column matrix. The column was allowed to stand for at least 3 minutes to ensures complete absorption of the elution solution. Finally, the column was centrifuged at 14,000 to 16,000 x g for 30 seconds to elute the purified DNA.

3.7 Polymerase Chain Reaction (PCR)

BoLA- DRB3 exon alleles from N= 20 cattles were analyzed by using the Gradient Thermal Cycler with their respective thermocycling conditions (Table 3.7.1) The use of specific PCR and

sequencing facilitates the identification of allelic variation within the peptide - binding cleft (PBC). The exon 2 region *BoLA - DRB3* gene was amplified using a single - step PCR (Baxter *et al.*, 2008), incorporating the middle set of primers employed by Miltiadou *et a*l., 2003 (Table 3.7.1).

A primer set (Table 3.7.1) made of intron sequences that flanks the *BoLA - DRB3* exon 2 were used for amplification (Miltiadou *et al*., 2003). These primers are designed to amplify a 284 bp fragment of exon 2 of the *BoLA - DRB3* gene (Mosafer *et. al*., 2012).

Table 3.7.1 Primer sets which are often referred to as *DRB3FRW* and *DRB3REV* are a well - established set specifically designed to amplify Exon 2 of the *BoLA - DRB3* gene.

Primers	Sequences
DRB3	**F**: 5' - CGC TCC TGT GAY CAG ATC TAT CC - 3' **R**: 5' - CAC CCC CGC GCT CAC C - 3'

PCR reactions were carried out in a final volume of 25 µl, in a 0.2 ml of PCR tubes, containing the components listed in Table 3.7.2.

Table 3.7.2 PCR components and their respective volumes per reaction.

Reagent	1X Unit (µl)
Nucleus Free Water (NFW)	**5.5**
GoTaq Green MasterMix	**12.5**
Forward + Reverse Primer	**1 + 1**
Sample/ Negative Control	**5**
Total	**25 µL**

Table 3.7.3 Primer sequences, thermocycling conditions for samples.

SNP	Primer	Thermocycling conditions	References
DRB3	**F** : 5' - CGC TCC TGT GAY CAG ATC TAT CC - 3' **R** : 5' - CAC CCC CGC GCT CAC C - 3'	94ºC for 2.5 mins 30 cycles: 94ºC for 30 secs 57ºC for 30 secs 72ºC for 1 min 72ºC for 5 mins 12ºC for ∞	Miltiadou *et al*., 2003

3.8 Agarose gel electrophoresis

PCR products were later analyzed by gel electrophoresis in a 1.5% agarose gel dissolved with 80 ml of TAE buffer and heated with microwave for 3 minutes of 100ºC until it liquified. Then, 1µl of Midori Green Advance was stained with the gel. As it cools, it solidifies into a porous matrix. The gel was run at 80 minutes for 80 voltages. The gel was visualized using Gel Doc ™ EZ Imager under UV trans - illuminator post-electrophoresis. Specific bands corresponding to the expected size of each PCR product were observed as positive results.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Demographic data of Cattle in Kelantan (Table 4.1.1 and Table 4.1.2)

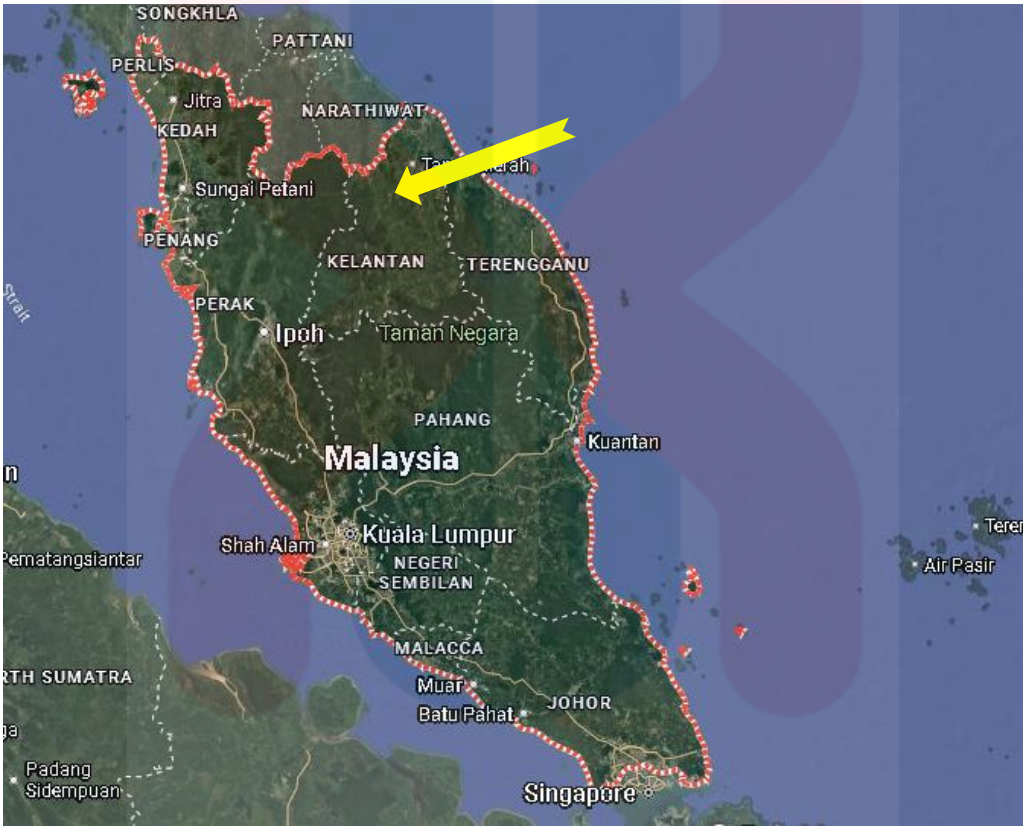

Figure 4.1.1 Demographic of Kelantan, Malaysia where the yellow arrow showing the location of both breeds were reared.

The demographic composition of cattle farming in Kelantan (Figure 4.1.1) is predominantly characterized by smallholder farmers who rear cattle as a supplementary income source. Kelantan, with approximately 94,615 cattle recorded in recent years, is one of Malaysia's most populous states and significantly contributes to the nation's beef industry (Radzil *et al*., 2023). The climate of Kelantan presents distinct advantages and challenges for cattle farming. This region possesses a tropical climate characterized by pronounced wet and dry seasons. The monsoon season, occurring from November to December, is characterized by elevated humidity and substantial

precipitation. Environment-sensitive diseases such as fascioliasis, which has been demonstrated to correlate positively with rainfall and humidity in Kelantan cattle herds, are more prevalent in this environment, which also fosters the proliferation of natural fodder (Faez *et al*., 2023). According to BERNAMA (2023), elevated temperatures and heat stress events can negatively affect cattle health and productivity, emphasizing the importance of management practice such as shade provision and adequate hydration.

Figure 4.1.2 Kelfarm Sdn. Bhd (6°03'11.2"N 102°16'04.3"E) was identified by using Google maps.

Figure 4.1.3 Miyazaki Wagyu in Kelfarm Sdn. Bhd, in Kota Seribong, Kelantan, Malaysia was reared in a fully intensive system and controlled environment.

Raising specialized cattle such as Miyazaki Wagyu, derived from the Japanese Black breed (Kuroge Wagyu), in Malaysia's tropical climate poses considerable difficulties. According to the Gotoh et al., 2018, Miyazaki Wagyu's success in Japan is closely related to its cold climate, pure habitat, and meticulous, traditional feeding methods. Adapting this breed for sustained, high-quality beef production in the hot, humid Malaysian climate necessitates specialist management and significant investment. Temperatures beyond these (4ºC to 20ºC) limits, particularly sustained exposure above 20°C, can induce heat stress, diminishing feed consumption and growth performance, hence adversely impacting marbling and meat quality. To mitigate heat stress in elevated temperatures, shading, ventilation, misting, and adequate hydration are essential management strategies.

Kelfarm is an intensive Wagyu cattle rearing system located in Kota Seribong, Kelantan, Malaysia (Figure 4.1.2 and Figure 4.1.3), that controls temperature and ventilation automatically to maintain optimal conditions for cattle growth and meat quality. The system maintains a maximum temperature of 24°C, ensuring the environment remains cool enough to minimize heat stress, which is critical for preserving Wagyu's signature marbling and tenderness. Automatic ventilation adjusts airflow as needed to maintain air quality and temperature, promoting cattle comfort and reducing respiratory and heat-related issues.

Table 4.1.1 Samples collected in Kelfarm Wagyu, Seribong, Kelantan including the health record as well for record keeping purposes.

No.	Tag number	Sex	Age	Details
1	202	Male	1 year 4 months	Feedlot, and vaccinated
2	206	Male	1 year 2 months	Feedlot, and vaccinated
3	207	Male	1 year 4 months	Feedlot, and vaccinated
4	221	Male	2 years 9 months	Feedlot, and vaccinated
5	217	Male	2 years 7 months	Feedlot, and vaccinated
6	126	Female	4 years 10 months	AI programed, and vaccinated
7	123	Female	4 years 11 months	AI programed, and vaccinated
8	132	Female	4 years 8 months	AI programed, and vaccinated
9	102	Female	4 years 9 months	AI programed, and vaccinated
10	150	Female	2 years	AI programed, and vaccinated

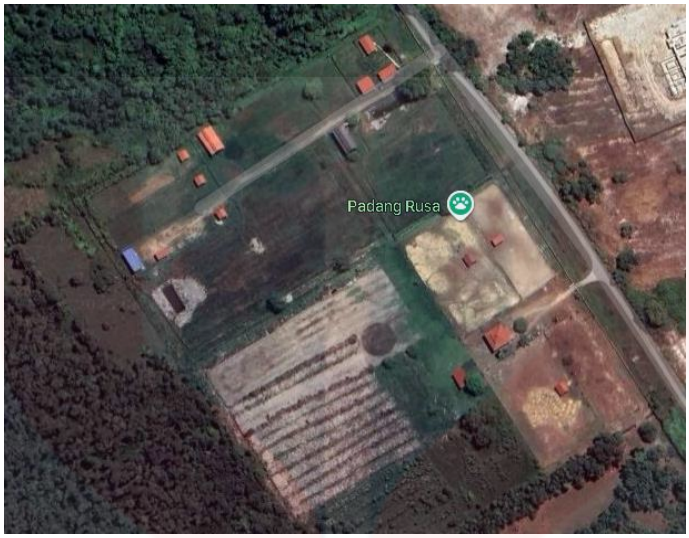

Figure 4.1.4 UMK Teaching Farm, UMK Bachok, Kelantan was identified by using Google maps.

Figure 4.1.5 KK cattle in UMK Teaching Farm, UMK Bachok were reared in an extensive system due to the high adaptability towards the environment.

The Kedah-Kelantan (KK) cattle of Malaysia are a resilient and well-adapted indigenous breed, mostly raised for meat production. According to Islam et al., 2022, they have a strong mothering ability and are extremely fertile. They are also genetically adapted to high temperatures, high humidity, and heavy rains like in the Figure 4.1.5, where they are usually reared in an extensive

system. Moreover, they exhibit excellent survival, possess resistance to prevalent illnesses, and can effectively consume low-quality native fodder with little housing requirements (Johari *et al*., 2009). About 90% of these cattle are raised with little feed supplementation in an extensive system (Jamaludin *et al*., 2014; Raymond *et al*., 2012). A study demonstrated variances in coat color among KK cattle, signifying the heterogeneity of the KK cattle population. The observed variability may result from selective preferences of each breeding site, as variable coat color is a favored characteristic among producers. The color of an animal's coat directly influences its capacity to endure environmental stressors, including heat stress, flies, and lice infestations (Burrow *et al.*, 2019). The prevalent coat color in KK cattle was brown (>82%), with a minority exhibiting different color patterns such as gray, black, or a combination of brown with white patches. The variance in coat color among animals is markedly affected by geographical location and environmental circumstances (Lü *et al*., 2016). To date, Islam et al., 2022 stated, KK cattle have exhibited black (73.9%) and brown (26.1%) skin colors; however, no comparative research exists regarding the skin colors of other cattle breeds.

Table 4.1.2: Samples collected in Teaching Farm of UMK Bachok, Kelantan including the record keeping for the health.

No.	Tag number	Sex	Age	Details
1	1202	Female	10 years	Given ivermectin, dewormed, vitamin, vaccinated
2	1203	Female	11 years	Given ivermectin, dewormed, vitamin, vaccinated
3	1204	Female	10 years	Given ivermectin, dewormed, vitamin, vaccinated
4	1205	Female	12 years	Given ivermectin, dewormed, vitamin, vaccinated
5	1208	Female	3 years	Given ivermectin, dewormed, vitamin, vaccinated
6	1210	Female	3 years	Given ivermectin, dewormed, vitamin, vaccinated
7	1214	Female	2 years	Given ivermectin, dewormed, vitamin, vaccinated
8	1226	Female	2 years	Given ivermectin, dewormed, vitamin, vaccinated
9	1229	Female	2 years	Given ivermectin, dewormed, vitamin, vaccinated
10	181	Female	2 years	Given ivermectin, dewormed, vitamin, vaccinated

4.2 Phenotypic findings

Figure 4.2.1 Miyazaki Wagyu in Kelfarm Sdn. Bhd with a body condition score (4 /5) based on Queensland Department of Agriculture of Fisheries (QDAF) 2019, cattle body condition scoring chart.

This exhibited a predominantly solid black coat colour, with minor visible markings or mud stains on certain parts of the body, and a body condition score (BCS) of 4/5, characterized by non – visible hip bones, well – covered ribs, a slightly lumpy tail head region, and a generally rounded body outline. Wagyu cows that calve with BCS of 5 tend to return to estrus sooner, produce more vigorous calves at birth, and yielded greater quantities of higher – quality colostrum compared to thinner cows (*Weaning*, 2021).

Figure 4.2.2 Kedah – Kelantan cattle in UMK Teaching Farm, UMK Bachok with a BCS 3/5 based on Queensland Department of Agriculture of Fisheries (QDAF) 2019, cattle body condition scoring chart.

This breed has a distinctive brown or reddish coat color. The body frame appears leaner and shorter compared to Wagyu, reflecting their adaptation to low – input tropical environments and traditional extensive production systems. Morphometric studies report that KK cattle possess comparatively modest body lengths, wither heights and girth measurements, indicative of a smaller breed conformation relative to larger beef breeds (Islam et al., 2022). Different from Miyazaki Wagyu, KK presented with BCS 3/5 where the hip bones visible faintly, ribs generally not visible, tail – head area not recessed and body outline almost smooth. This BCS may be relates with the management of KK. These differences in body frame and growth performance underscore the divergent selection pressures between indigenous adaptive breeds like KK and specialized meat breeds such as Wagyu (Islam et al., 2022).

4.3 Determination of Quality and Quantity of DNA

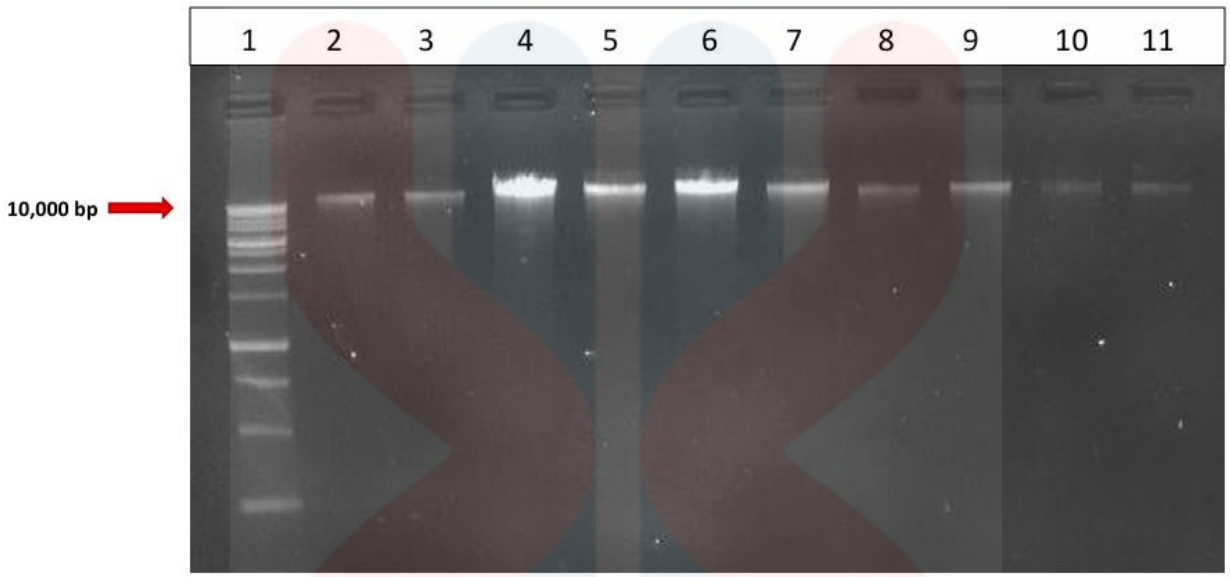

Figure 4.3.1 Optimization of *Taq* DNA Polymerization concentration for Miyazaki Wagyu cattle.
Lane 1: 1Kb DNA ladder
Lane 2 - 11: Miyazaki Wagyu samples

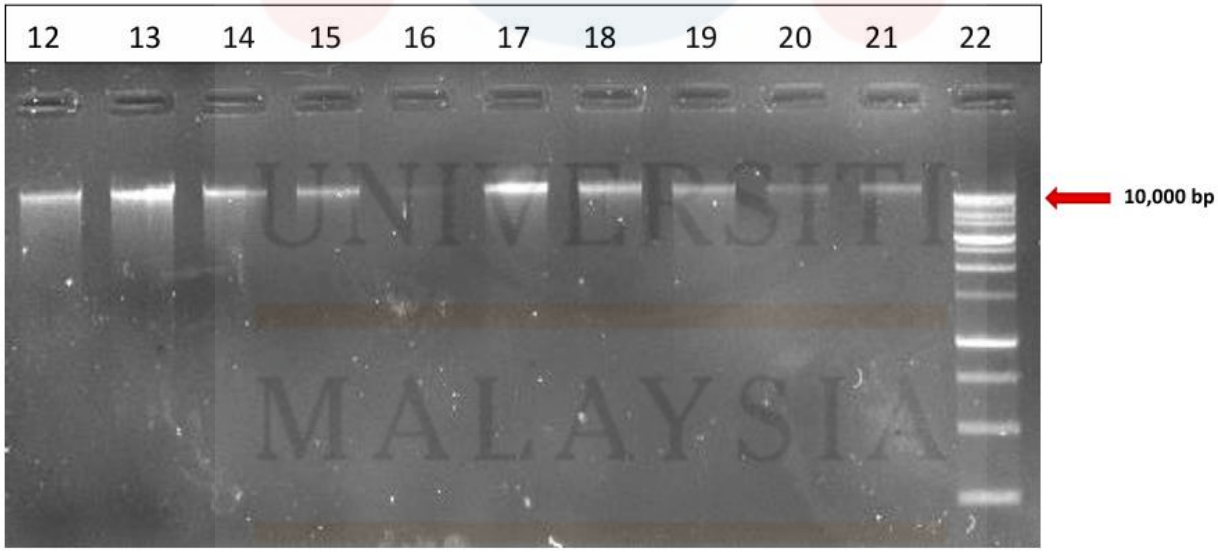

Figure 4.3.2 Optimization of *Taq* DNA Polymerization concentration for Kedah - Kelantan (KK) cattle.
Lane 22: 1Kb DNA ladder
Lane 12 - 21 : KK samples

Both breeds, Miyazaki Wagyu and KK cattle show distinct bands at the expected sizes. DNA purity is evaluated on a gel by the appearance of intact bands especially in comparison to a DNA ladder like the Promega 1kb ladder.

4.4 Result of PCR

These primers (Table 3.7.1) are specifically designed to amplify the entire Exon 2 of the *BoLA - DRB3* gene, which encodes the highly polymorphic peptide binding region. Theoretical size for the Exon 2 target sequence is ~284 bp (Mosafer et al., 2012).

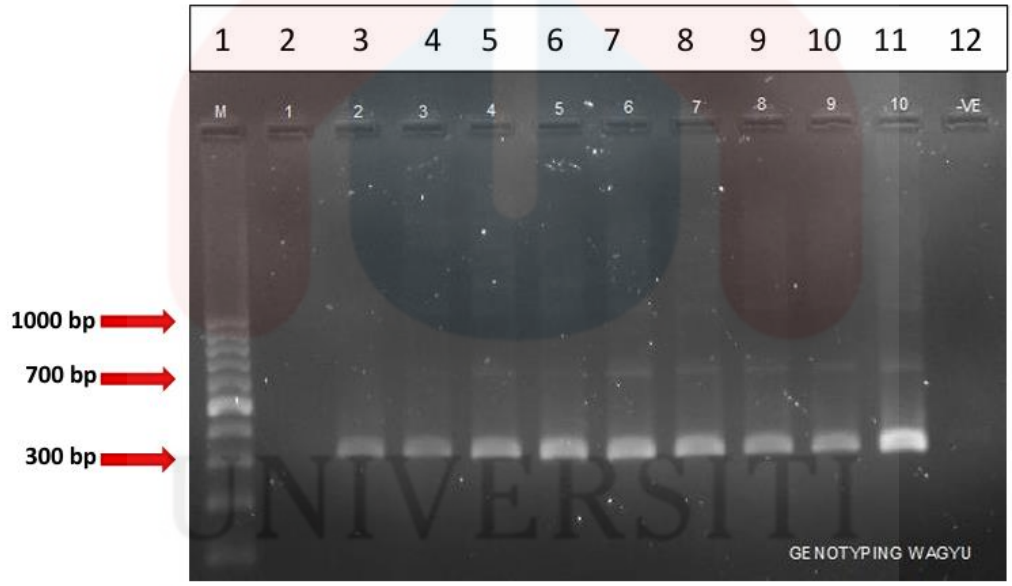

Figure 4.4.1 Gel electrophoresis image showing amplification of *DRB3* gene. Single clear bands indicate positive results in Miyazaki Wagyu cattle.

Lane 1: 1000 bp ladder
Lane 2 - 11 : Miyazaki Wagyu samples
Lane 12 : Negative control

Table 4.4.1 Summary of the gel electrophoresis image for Miyazaki Wagyu that shows amplification of *DRB3* gene. Single clear bands indicate positive results. A total of 10 samples were taken from Miyazaki Wagyu cattle, labelled 2 - 11.

Miyazaki Wagyu cattle			
Lane	**Sample ID**	**Band size**	**Interpretation**
1(M)	Ladder	1,000 bp	DNA size marker for size reference only
2	1	No band	Possible no amplification or very low concentration
3	2	~ 300bp	Clear positive amplification
4	3	~ 300bp	Clear positive amplification
5	4	~ 300bp, ~700bp	Clear positive amplification
6	5	~ 300bp, ~700bp	Clear positive amplification
7	6	~ 300bp, ~700bp	Clear positive amplification
8	7	~ 300bp, ~700bp	Clear positive amplification
9	8	~ 300bp, ~700bp	Clear positive amplification
10	9	~ 300bp, ~700bp	Clear positive amplification
11	10	~ 300bp, ~700bp	Clear positive amplification
12	Negative control	No band	Expected result with no band

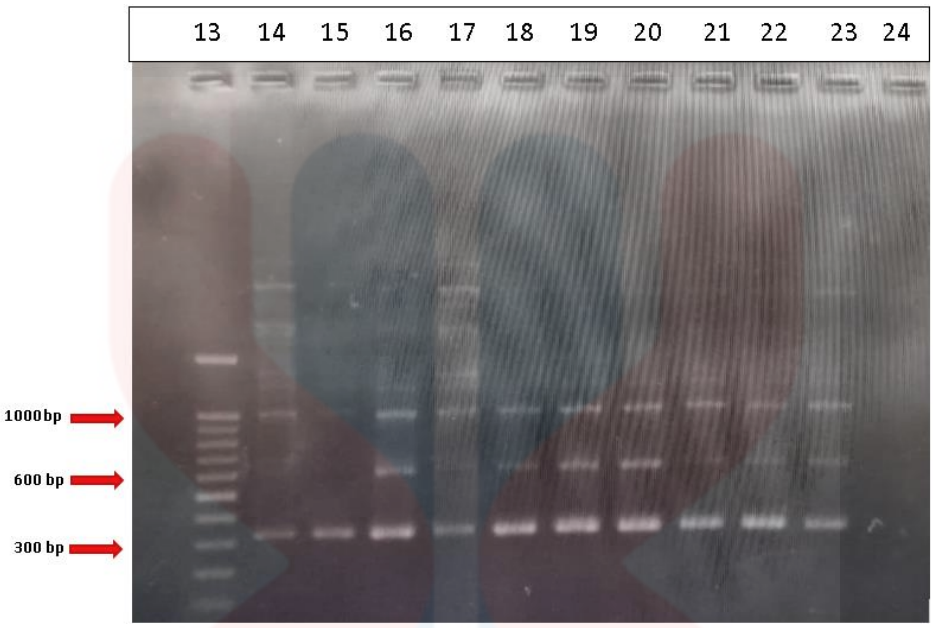

Figure 4.4.2 Gel electrophoresis image showing amplification of *DRB3* gene. Single clear bands indicate positive results in Kedah - Kelantan (KK) cattle.

Lane 13: 1000 bp ladder
Lane 14 - 23: KK samples
Lane 24 : Negative control

Table 4.4.2.1 Summary of the gel electrophoresis image for KK cattle that shows amplification of *DRB3* gene. Single clear bands indicate positive results. A total of 10 samples were taken from Kedah - Kelantan cattle, labelled 14 - 23.

Kedah - Kelantan (KK) cattle

Lane	Sample ID	Band size	Interpretation
13 (M)	Ladder	1,000 bp	DNA size marker for size reference only
14	11	~300bp	Clear positive amplification
15	12	~300bp	Clear positive amplification
16	13	~300bp, ~600bp	Clear positive amplification
17	14	~300bp, ~600bp	Clear positive amplification
18	15	~300bp, ~600bp	Clear positive amplification

19	16	~300bp, ~600bp	Clear positive amplification
20	17	~300bp, ~600bp	Clear positive amplification
21	18	~300bp, ~600bp	Clear positive amplification
22	19	~300bp, ~600bp	Clear positive amplification
23	20	~300bp, ~600bp	Clear positive amplification
24	Negative control	No band	Expected result with no band

The amplification of BoLA – DRB3 exon 2 region was successful in 9 out of 10 Miyazaki Wagyu samples and all 10 KK cattle samples, as shown in Figure 4.4.1 and Figure 4.4.2. The predominant and expected amplicon size was approximately 300 bp, visible as a clear, single band in the majority of the lanes. This observed size is consistent with the reported range of 279 bp to 320 bp for this primer set (Miltiadou et al., 2003; Agina et al., 2023), with the slight deviation from the theoretical 284 bp falling within the standard margin of error for agarose gel electrophoresis (Sambrook & Russel, 2001).

Notably, several samples from both breeds exhibited additional, fainter bands at higher molecular weights (~ 700 bp in Miyazaki Wagyu sample 4 – 10; ~ 600 bp in KK cattle samples 13 - 20). These secondary bands are a common observation in PCR of highly polymorphic loci like *BoLA – DRB3* and may represent heteroduplex formation between different allelic sequences, non – specific priming, or the amplification of related genomic regions. Their specific nature cannot be determined without sequencing.

Table 4.4.2.2 For the detection of specific blood pathogens using their respective primers, the expected fragment (band sizes) were as follows :

Pathogen	Band size (bp)	Reference
Trypanosoma evansi	205	Agina et al. (2023)
Candidatus Mycoplasma haemobos	279	
Anaplasma platys	540	
Anaplasma marginale	761	
Theileria orientalis	776	

4.5 Discussion

This study successfully established and validated a PCR assay for the consistent amplification of exon 2 of the *BoLA – DRB3* gene in two distinct cattle populations in Kelantan, Malaysia. The generation of a clear ~300 bp amplicon in 19 out of 20 samples (95%) confirms the specificity of the primer set and the robustness of the optimized thermocycling conditions. This foundational step is critical, as it verifies the presence and accessibility of this key immunogenetic locus, thereby enabling all subsequent high – resolution analyses aimed at understanding genetic diversity and disease resistance.

The near universal amplification success in the indigenous KK cattle (10 out of 10) aligns with expectations for a *Bos indicus* breed shaped by long – term natural selection. Evolving under the endemic pressure of tropical hemoparasites such as *Theileria* and *Anaplasma* spp. in the Malaysian environment, KK cattle are hypothesized to maintain substantial polymorphism at immune – response genes like *BoLA – DRB3* as a mechanism of adaptive resistance (Rahman et al., 2020). In contrast, the single amplification failure in one Miyazaki Wagyu sample is most parsimoniously explained by technical factors, such as suboptimal DNA concentration. However, this result invites

consideration of the breed's distinct genetic history. Miyazaki Wagyu are a *Bos taurus* breed subjected to generations of intensive, closed – population selection in Japan for supreme marbling, a process historically opposite from pathogen – driven natural selection (Miyazaki et al., 2018). While the sampled Miyazaki Wagyu cattle were reared in Thailand prior to import, a region with a broadly similar tropical climate to Kelantan where such individual exposure provides immunological priming but is unlikely to have instilled the deep, heritable allelic diversity that arises from centuries of co – evolution with local pathogen strains. Plus, with the fully intensive system, the cattle has lesser exposure towards the environment and makes them more susceptible towards endemic diseases. Consequently, Miyazaki Wagyu populations are documented to have reduced genetic diversity at MHC loci, potentially increasing their vulnerability to novel or endemic diseases in new environments as well (Takeshima & Aida, 2006; Olsen et al., 2018).

An intriguing observation from the gel electrophoresis was the consistent presence of secondary, higher molecular weight bands in several individuals from both breeds (~ 700 bp in Miyazaki Wagyu from sample 4 to 10; ~600 bp in KK cattle samples from sample 13 to 20). In PCR of highly polymorphic loci, such bands commonly represent technical artifacts like heteroduplex complexes formed between different allelic sequences during amplification. A phenomenon itself suggestive of heterozygosity. Their consistent size, however, prompts further speculative consideration. Fragment sizes in this range are reported for common endemic hemoparasites like *Anaplasma platys* (540 bp) and *Theileria orientalis* (~776 bp) (Agina et al., 2023). It is imperative to clarify that this study did not perform pathogen – specific PCR. Therefore, these bands cannot be diagnostically interpreted. Their presence nevertheless underscore the complex microbial environment in which these cattle live and reinforces the biological relevance of studying the *BoLA*

– *DRB3* gene in this context. The definitive identity of these secondary amplicons should be investigated in future work via targeted pathogen PCR.

A core limitation of this gel – based assay must be explicitly stated as while PCR confirms the presence of the *BoLA – DRB3* gene, it does not identify alleles or quantify genetic diversity. Its primary contribution is providing a validated, reliable PCR protocol that paves the way for the essential next steps which are the allelic characterization and phenotypic correlation. To directly test the hypothesis that KK cattle possess greater immunogenetic diversity conferring enhanced disease resilience, future research must employ sequence – based typing like Sanger sequencing, PCR – RFLP to identify specific *BoLA – DRB3* alleles. Subsequently, these genotypic data must be correlated with phenotypic data on health and pathogen load. Performing concurrent diagnostic screening for key endemic hemoparasites on the same cohort will enable robust statistical analysis to identify alleles associated with resistance or susceptibility.

In conclusion, the hypothesis that the *BoLA – DRB3* gene can be detected in both KK and Miyazaki Wagyu cattle via PCR is strongly supported. The results align with the broader narrative that indigenous breeds represent reservoirs of genetic diversity shaped by environmental pressures, while specialized breeds reflect the trade – offs of intensive artificial selection. By successfully bridging the gap between sample collection and advanced genetic analysis, this study confirms that the immunogenetic blueprint of these breeds is accessible for deciphering. It sets a clear and critical trajectory for research that can ultimately link specific *BoLA – DRB3* alleles to the documented resilience of KK cattle and inform breeding or management strategies to mitigate potential vulnerabilities in exotic breeds within Malaysia's tropical livestock sector.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This study has successfully genotyped the *BoLA - DRB3* exon 2 locus in both breeds by using a PCR assay. Although the study did not extend to allele level characterization, the PCR results have provided a foundation for evidence that the gene is amplifiable and suitable for further genetic analysis.

This detection provides farmers with practical insights into managing and rearing cattle based on breed specific strengths. By identifying cattle that carry favorable immune related traits, farmers can gradually develop herds that are healthier and more resilient to common diseases in Malaysia such as mastitis, tick borne infections and blood parasites infections other than management causes. Cattle with stronger genetic resistance experience fewer treatment dependent illnesses, which in turn reduces veterinary costs, labour needed, and overall economic losses. Over time, improved herd health and productivity may also contribute to increasing the national beef SSL and reducing reliance on imported cattle.

Overall, this research contributes to the baseline molecular data that can support future genetic improvement programs and provide direction for enhancing cattle disease resilience in Malaysia's tropical production system and with that the hypothesis has been achieved.

5.2 Recommendation for future work

The recommendations of this study are to perform allele typing using sequencing or PCR - RFLP etc. This is because PCR detection alone is unable to identify the allelic variants, level of genetic diversity and specific variants associated with disease resistance or susceptibility of *BoLA - DRB3*. Additionally, it is advisable to increase the sample size across several farms and areas to improve the statistical robustness and address regional variability especially in KK cattle, whose genetic composition may different by locality.

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