ASSESSMENT OF DNA MARKERS FOR SEX DETERMINATION IN MALAYSIAN CATTLE

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ABSTRACT. In Malaysia, the economic value and productivity of cattle in the meat and dairy sectors are heavily influenced by sex, with males preferred for meat production and females for dairy. However, accurate sex determination methods for cattle remain underdeveloped, necessitating research into reliable genetic markers. To our knowledge, this is the first study in Malaysia to validate proteolipid protein (PLP) and glycoprotein M6B (GPM6B) as highly accurate X-linked markers across diverse cattle breeds. This study aims to identify specific genetic markers, including proteolipid protein (PLP), glycoprotein M6B (GPM6B), sex-determination region Y (SRY), and testis-specific protein, Y-encoded (TSPY) for accurate sex determination of local cattle and to assess the accuracy and sensitivity of PCR-based techniques. Samples from various cattle breeds were analysed using DNA extraction from blood and PCR amplification targeting sex-specific genetic markers. Results were assessed through gel electrophoresis. X-linked markers (PLP and GPM6B) demonstrated 100% accuracy, sensitivity, and specificity in identifying female DNA, making them robust tools for sex determination. Y-linked markers (SRY and TSPY) showed slightly lower accuracy (97.73% and 98.88%, respectively) due to occasional inconsistencies. These findings highlight the potential for integrating molecular tools into breeding programs. This study establishes the reliability of PLP and GPM6B markers for precise cattle sex determination, offering significant improvements in livestock management and breeding efficiency. Further research is recommended to refine protocols, reduce costs, and expand validation across different samples and diverse populations, supporting the sustainability of Malaysia's cattle industry.

Keywords: proteolipid protein, glycoprotein M6B, sex markers, blood, PCR

INTRODUCTION

In Malaysia, cattle are raised for both meat and milk production. In recent years, farmers have increasingly sought to enhance productivity through sex selection, as the economic value and breeding strategies are significantly influenced by the sex of the animals. Specifically, for dairy purposes, milk yield depends on the performance of female cattle, whereas for meat production, males typically produce more meat than females.

According to the Department of Veterinary Services (DVS), in 2022, there were 259,926 males and 376,409 females beef cattle in Peninsular Malaysia. In contrast, the dairy sector was significantly lower, with 6,265 males and 25,204 females across all age groups. Overall, these figures suggest that the cattle population in Peninsular Malaysia is still relatively low for both the meat and dairy sectors.

Notably, in the dairy sector, most cattle are female primarily due to their key roles in reproduction and milk production. Female cattle are kept to maintain herd size and ensure a steady milk supply, while excess males are often sold or culled. This trend is common in dairy farming, where milk production is prioritized over meat

production. Moreover, in Peninsular Malaysia, dairy production is prioritized, as indicated by the higher number of dairy cattle, which reflects market-driven demand. Nevertheless, the overall cattle population remains low, demonstrating a reliance on imports and highlighting opportunities for expansion in the local beef and dairy industries. Encouragingly, the adoption of reproductive technologies, such as sexed semen and embryo transfer, has led to an increase in the number of female calf births, contributing to improved herd structure and enhanced profitability (Sagwa *et al*., 2019).

Sex determination, an assisted reproductive technology (ART), holds great potential for manipulating the sex ratio in cattle herds. However, the methods for determining animal sex are not yet well understood. The genetic and physiological foundations of sex selection remain unresolved across animal species, and genetic diversity among breeds can complicate sex determination. Despite these limitations, the use of DNA markers for selecting traits in cattle has greatly improved the efficiency of breeding programs. PCR-based methods, in particular, have shown high accuracy and sensitivity in sex determination, outperforming traditional hormone-based techniques. The SRY gene is widely used in these methods to quickly distinguish between male and female cattle by amplifying specific DNA fragments (Kőszegi *et al*., 2025; Tran *et al*., 2023). Both maternal plasma-based and direct DNA sampling methods have been shown to be effective (Tran *et al*., 2023; Nix *et al*., 2023). Furthermore, improvements in SCAR (Sequence Characterized Amplified Region) markers have further increased the accuracy of sex identification, especially when physical differences are unclear (Ahmad *et al*., 2021; Rahman *et al*., 2022). Collectively, these molecular tools support genomic selection for traits like meat quality and fertility (Kawaguchi *et al*., 2020; Sasazaki, 2021; Ceccobelli *et al*., 2022).

Numerous studies have demonstrated the use of gene markers in determining the sex of cattle, highlighting the accuracy and reliability of specific genetic sequences for identifying male and female specimens. The effectiveness of the SRY gene in polymerase chain reaction (PCR) methods for rapid sex determination has been well established, enabling precise identification without requiring whole-genome sequencing (Rahman *et al*., 2022; Ahmad *et al*., 2021). In addition, the amelogenin gene has been widely utilized as a key marker due to size differences in the resulting PCR products, with variations between X-linked AMELX and Y-linked AMELY aiding in sex differentiation (Ahmad *et al*., 2021).

Recently, studies on sex determination in cattle increasingly rely on genetic markers for their accuracy and consistency. PCR-based methods utilizing the SRY and amelogenin (AMEL) genes have proven effective for quickly and reliably identifying sex, aiding breeding programs and herd management (Rahman *et al*., 2022; Ahmad *et al*., 2021). The SRY gene targets male-specific DNA, while size differences in AMEL gene fragments on the X and Y chromosomes enable clear differentiation (Ahmad *et al*., 2021). Additionally, RNA-sequencing has expanded non-invasive options by enabling fetal sex identification through maternal blood analysis (Nix *et al*., 2023; Tran *et al*., 2023). Altogether, these advanced molecular tools enhance breeding efficiency and support genetic enhancement of valuable traits in cattle (Nix *et al*., 2023; Tran *et al*., 2023).

Despite significant advancements, several challenges persist in implementing sex determination techniques in local cattle, including the high costs associated with advanced technologies such as flow cytometry for sperm sorting and PCR-based methods for embryo sexing. These costs may restrict

accessibility in developing regions. Traditional methods like ultrasound fetal scanning and fetal DNA detection are often only applicable after pregnancy is established, restricting proactive management decisions for producers. Despite these challenges, there are significant opportunities for future research, particularly in optimizing low-cost DNA extraction methods and enhancing the efficiency of sex determination protocols, as demonstrated by the successful application of the HotShot alkaline lysis method (Tavares *et al*., 2016). Looking ahead, advancements in genomic tools and molecular markers can further improve the accuracy of selection and increase genetic gains in livestock production, addressing both economic and sustainability goals in the industry (Singh *et al*., 2019).

To our knowledge, this is the first study in Malaysia to validate PLP and GPM6B as highly accurate X-linked markers across diverse cattle breeds. Therefore, this study aims to identify specific genetic markers (e.g., PLP, GPM6B, SRY, TSPY) that can accurately determine the sex of local cattle and to evaluate the accuracy and sensitivity of PCR-based methods in detecting sex-specific markers. This research will contribute to enhancing the productivity and economic viability of cattle breeding programs in Malaysia.

MATERIALS AND METHOD

Primer design

Primers for the amplification process were designed based on the *Bos taurus* species from Genbank (http://www.ncbi.nlm.nih.gov). These primers were specifically developed to detect Y and X-bearing chromosomes, which are essential for sex selection. The primers were analyzed using the Primer 3 Plus program (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). A list of the primers is provided in Table 1. The simulation of the primers was conducted using the primer BLAST tool in GenBank (http://www.ncbi.nlm.nih.gov).

Sample Collection

The animals included in this study were sourced from nucleus herd farms managed by the Department of Veterinary Services (DVS). These farms include Pusat Ternakan Haiwan Air Hitam in Johor, which specializes in the Mafriwal dairy breed; Pusat Ternakan Haiwan Ulu Lepar in Kuantan, Pahang, which houses the Brahman breed; and Pusat Ternakan Haiwan Tersat in Terengganu, where the Kedah-Kelantan breed is maintained.

This study examines two distinct groups of cattle DNA. The first group consists of the dairy

Table 1: Genes involved in sex determination in cattle blood

Gene	Accession no. (NCBI)	Primer	Primer sequence (5'-3')	Annealing temperature	PCR product
PLP	NC_037357.1	F1	GTCATCTATGGAACTGCCTCTT	58	199
		R1	GCTCCAAAGAATGAGCTTGATG		
GPM6B	NM_001104981	F1	AGTCGGGAAGATTGCTGCACTA	58	290
		R1	TTTCAGTGGCTGCTCACCAA		
SRY	NM_001014385.1	F1	ATTAAGCCGGTCACAGTCCGT	53	119
		R1	AGTCCGCCGAAATCCGTGTA		
TSPY	x74028.1	F1	CCATTACGCCCCGACTTGCT	58	102
		R1	TCTGACCGCCTCTCACGTTC		

breed, Mafriwal cattle, while the second group comprises of beef breeds, including Brahman, Brahman cross, Nelore, and Kedah-Kelantan cattle.

Blood samples analysed in this study were obtained from farms operated by the Department of Veterinary Services as part of a routine herd health program that was independent of this research. Sample collection was performed by qualified personnel in accordance with standard veterinary health monitoring procedures, and no animals were specifically selected or sampled for research purposes.

DNA extraction from blood

A total of 10 mL of blood samples were collected from either the tail vein or jugular vein using ethylenediaminetetraacetic acid (EDTA) tubes. DNA was extracted from 200 µL of blood, processed using a commercial DNA Extraction Kit (Qiagen, USA). The extracted DNA was properly labelled and stored at -20°C for analysis.

Polymerase chain reaction

The oligonucleotide sequences of the primers, listed in Table 1, were utilized. The PCR reaction mixture had a total volume of 25 µL, containing 1× Gotaq Green Master Mix (Promega, USA), 0.5 µM of each primer, and 2.0 µL of DNA templates. PCR was conducted using a thermocycler (Applied Biosystems, California) under the following conditions: initial denaturation at 95°C for 2 minutes, followed by 38 cycles with denaturation at 95°C for 45 seconds, annealing at 53–58°C for 45 seconds, and extension at 72°C for 60 seconds. A final extension at 72°C for 5 minutes completed the process. Each sample was run in duplicates to ensure the reliability of the results.

Table 2: The detection results of PLP, GPM6B, SRY, and TSPY amplification in cattle breeds using the PCR technique

Breed	Sex	Recorded Sex of Cattle by Farm Records, N	Electrophoresis Result			
			Chromosome X		Chromosome Y	
			PLP	GPM6B	SRY	TSPY
Brahman	Male, XY	5	5	5	5	5
	Female, XX	11	11	11	0	0
Brahman cross	Male, XY	3	3	3	3	3
	Female, XX	0	0	0	0	0
Nelore	Male, XY	5	5	5	5	5
	Female, XX	5	5	5	0	0
Kedah-Kelantan	Male, XY	12	12	12	12	12
	Female, XX	18	18	18	1*	1*
Mafriwal	Male, XY	10	10	10	9*	10
	Female, XX	20	20	20	0	0

Note: *The sex determined by the testing method did not align with the recorded sex in the farm's documentation.

Gel electrophoresis and analysis

The PCR products were analysed using 3% agarose gel electrophoresis in 1× TBE buffer, run for 90 minutes at 90 volts. The gels were stained with Fluorosafe (1stBase, Singapore) and visualized under UV light using a transilluminator. The sizes of the PCR fragments were determined using Bio-Print software (Vilber, France).

RESULTS & DISCUSSION

Figure 1A to 1D illustrates the results of DNA-based sex determination in 89 cattle (36 males and 53 females) using conventional PCR techniques. Blood samples were analysed for the presence of the PLP, GPM6B, SRY, and TSPY genes. As shown in Table 2, sex determination across four cattle breeds can be achieved using these markers.

According to Table 3, PLP and GPM6B exhibit 100% accuracy, sensitivity, and specificity for both male and female samples, confirming their reliability for precise X chromosome identification. In contrast, SRY and TSPY, which target the Y chromosome, display slightly lower accuracy (97.73% and 98.88%, respectively) and specificity (98.15% for both), indicating occasional errors in sex differentiation. SRY showed slightly reduced sensitivity, possibly due to primer inefficiency or DNA degradation (97.14%), which is likely attributed to challenges in amplifying or detecting the Y chromosome in specific samples.

Overall, both PLP and GPM6B markers are exceptionally reliable for distinguishing between male and female cattle, demonstrating high accuracy, sensitivity, and specificity in sex determination. The PLP gene, primarily known

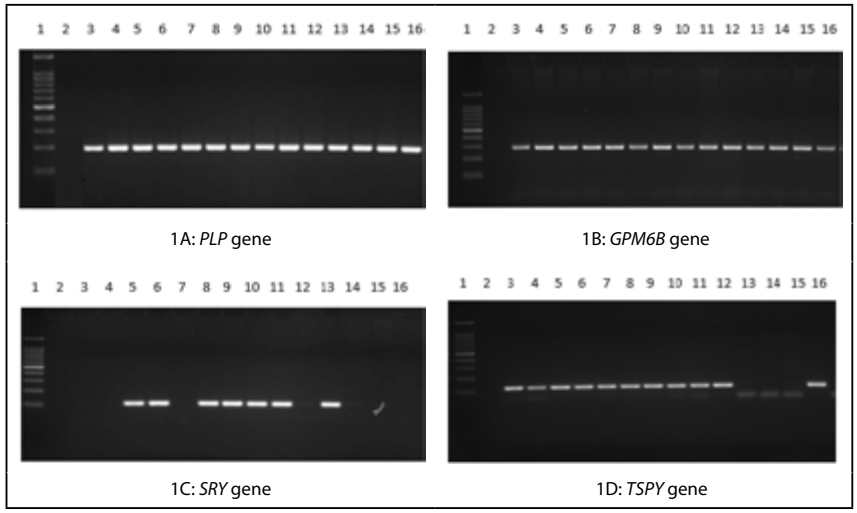

Figure 1. PCR analysis of X- and Y-linked genes in cattle. The figure illustrates the results of agarose gel electrophoresis on PCR products from cattle blood samples. As shown in panels 1A and 1B, the X-linked genes *PLP* and *GPM6B* were expressed in all male and female samples, revealing bands at 199 base pairs (bp) and 290 bp, respectively. In contrast, the Y-linked genes *SRY* and *TSPY* (panels 1C and 1D) were expressed only in male samples, with corresponding bands at 119 bp and 102 bp. In each gel, lane 1 contains the DNA ladder for size reference, and lane 2 serves as the non-template control, which consists only of double-distilled water to ensure no contamination. Lanes 3 through 16 correspond to the experimental samples.

Table 3: Evaluation of blood sample accuracy, sensitivity, and specificity using conventional PCR method with PLP, GPM6B, SRY, and TSPY genes

Metric	PLP		GPM6B		SRY		TSPY	
	Male 35	Female 54	Male 35	Female 54	Male 35	Female 54	Male 35	Female 54
Accuracy	100%		100%		97.73%		98.88%	
Sensitivity	100%		100%		97.14%		100%	
Specificity	100%		100%		98.15%		98.15%	

for its role in the central nervous system, is also linked to sex determination in bovine species. Located on the X chromosome, PLP has been studied in the context of sex determination. For instance, Khirwat (2023) developed a qPCR assay targeting both PLP and SRY genes to determine sperm sex ratios in Indian cow bulls, achieving high accuracy and reliability. This finding underscores the utility of PLP as a complementary marker to SRY in reproductive genetics. Similarly, Harshini *et al*. (2022) indicated that the PLP marker could determine the sex ratio of bovine semen using qPCR in spermatozoa.

Beyond sex determination, the PLP gene has been associated with body size traits in cattle, suggesting its potential influence on growth and development (Zhou *et al*., 2019). Recent research suggests a possible link between the proteolipid protein 1 (PLP) gene and body size traits in cattle, however the evidence remains limited. Genome-wide association studies have identified SNPs related to size traits, with some studies implicating regions near PLP (Abdalla *et al*., 2023; Chen *et al*., 2020; Garavito *et al*., 2023). While it has not been a central focus, PLP's potential role in growth traits warrants further investigation to clarify its function. In comparison, the GPM6B gene, although less frequently referenced in sex determination, has been studied in cattle genetics due to its conservation across breeds. This conservation suggests a potential role in critical phenotypic traits, although specific diagnostic studies remain limited (Cui, 2023). Consequently, the conserved regions within GPM6B indicate its functional significance in cattle genetics, warranting further investigation into its role in reproductive traits.

Turning to the Y-linked markers, the SRY gene, located on the Y chromosome, is essential for male sex determination in mammals, including cattle. It is susceptible and specific in detecting male DNA. Giguère *et al*. (2019) demonstrated that the TSPY gene, also on the Y chromosome, could identify male DNA at much lower concentrations than the SRY gene, indicating its superior sensitivity in detecting male genetic material. This study aligns with TSPY's role in male fertility and sex determination (Kolasa *et al*., 2015). Wang *et al*. (2010) found that the SRY gene could predict fetal sex by amplifying fetal DNA in cow plasma, demonstrating high accuracy in identifying male embryos. Other studies support the use of SRY in prenatal diagnostics, reporting nearly 100% specificity in detecting male fetuses through maternal plasma analysis (Fernández-Martínez *et al*., 2012).

CONCLUSION

The study successfully identified and validated specific genetic markers for sex determination in local cattle using blood samples, thereby contributing to advancements in livestock

management and enhancing productivity. Among the markers evaluated, the X-linked genes PLP and GPM6B proved highly reliable, demonstrating perfect accuracy, sensitivity, and specificity in identifying female DNA. These markers showed strong consistency across diverse cattle breeds and developmental stages, reinforcing their applicability for precise sex determination. Conversely, the Y-linked markers SRY and TSPY exhibited slightly lower performance due to occasional inconsistencies in amplification. Nevertheless, their role in detecting male DNA highlights their potential in breeding programs designed to optimize sex ratios.

While the findings highlight the viability of molecular approaches in cattle breeding, several technical challenges remain, including inconsistencies in detecting specific markers. Addressing these issues through improved PCR protocols, higher-quality DNA templates, and broader validation across breeds is essential to enhance the effectiveness of these techniques.

In conclusion, this research demonstrates that integrating molecular tools into livestock breeding programs has the potential to enhance efficiency, productivity, and overall economic viability substantially. The adoption of reliable markers, such as PLP and GPM6B, is a transformative step toward sustainable agricultural practices. Future efforts should focus on refining methodologies and scaling these innovations to meet the increasing demands of the Malaysian meat and dairy industries, as well as those in other regions.

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