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Genetic Diversity of the *Agouti Signaling Protein* (*ASIP*) Gene in Determining Coat Color in Bali Cattle (*Bos javanicus*)

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ABSTRACT

Bali cattle, which are indigenous to Indonesia, display distinctive and unique coat color characteristics. The appearance of albinos has been observed as an abnormality. The present study aimed to explore the *agouti signaling protein* (*ASIP*) gene in Bali cattle and analyze its association with the occurrence of albinism and white-spotted coat colors in Bali cattle. A total of 68 blood samples from cattle were used, including standard Bali cattle (N = 39), Bali cattle with spotting (N = 9), and albino Bali cattle (N = 20). Standard and white-spotted Bali cattle samples were collected from the Bali Cattle Breeding Centre in Jembrana, Bali Province, and the Breeding Center Unit in Serading, West Nusa Tenggara, Indonesia. Albino cattle samples were obtained from Taro Village, Gianyar Regency, Bali. The DNA amplification was performed using polymerase chain reaction (PCR), single-nucleotide polymorphisms (SNPs) were determined through direct sequencing, and genotyping was performed using the PCR-RFLP method. The exon 1 and exon 2 regions of the *ASIP* gene were monomorphic or uniform. The coding region of exon 3 of the *ASIP* gene in Bali cattle exhibited polymorphisms, specifically regarding coat color. A novel SNP (g.498 A > G) was detected exclusively in Bali cattle; however, it has not yet been validated as a potential genetic marker for coat color in this breed. The findings of this study further revealed that the *ASIP* gene sequence does not distinguish between standard and albino coat colors in Bali cattle, despite the identification of this specific SNP.

Keywords: *Agouti signaling protein* gene, Bali cattle, Coat color, Polymerase chain reaction, Single nucleotide polymorphism

INTRODUCTION

Bali cattle (*Bos Javanicus*) are a native Indonesian breed distinguished by their unique coat color characteristics. The coat is black in adult males, whereas females exhibit a brick-red hue. The lower part of the legs displays a white coloration resembling socks, and a crescent-shaped white patch is present on the buttocks. However, recent studies have identified coat colors that deviate from the usual characteristics, giving rise to albino Bali cattle (Reza et al., 2024). Albinism results from a lack of melanin in the skin, eyes, and hair (Ma and Wang, 2024). Albinism in mammals is an autosomal recessive condition that leads to abnormalities in eye, hair, and skin pigmentation (Zhao et al., 2015).

A 7.63% albino case rate in the Kupang region of Indonesia, while albino Bali cattle cases found in their native breeding area (Bali Province) amounted to 0.33% (Heryani et al., 2018). The discovery of albino Bali cattle is crucial, as it may impede the purification efforts of Bali cattle as a native Indonesian breed. Additionally, the emergence of albino Bali cattle poses a challenge in Bali cattle breeding, particularly in maintaining the purity of superior breeding males that will be distributed to the community (Purwantara et al., 2012). The uncontrolled increase in the population of albino Bali cattle can also lead to the extinction of pure Bali cattle breeds in Indonesia (Septian et al., 2015). Therefore, to preserve and maintain the genetic purity of Bali cattle, it is important to conduct studies that analyze the causes of albinism in this breed. Such studies can take advantage of advanced molecular genetic technologies, particularly marker-assisted selection (MAS), which allows for the identification of gene polymorphisms associated with albino traits. In general, coat color is determined by genetic factors (Gao et al., 2017).

Various genes, including *melanocortin 1 receptor* (*MC1R*), *receptor tyrosine kinase* (*KIT*; dominant white), *TYRP1* (brown), *KITLG* (roan), *MITF* (white-spotted), *TYR* (albinism), and *Agouti Signaling Protein* (*ASIP*; agouti), have been identified as influencing coat color in cattle (Schmutz, 2012). Analysis revealed the presence of Single Nucleotide

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Polymorphisms (SNPs) in the partial intron 2 and exon 3 regions of the *KIT* gene in Bali cattle (Jakaria et al., 2023). However, these SNPs cannot be used as standardized markers for coat color, white spotting, or albinism in Bali cattle. In addition, the MC1R gene was monomorphic. Some cattle exhibit primary pigmentation in red and black (Angus cattle; He et al., 2022), whereas others display a white coat (Charolais cattle; Gutiérrez-Gil et al., 2007). Colors such as spotting, dilution, roan, and brindle are influenced by various genes. Pigmentation in coat color is a crucial factor in livestock breeding, as it impacts productivity and adaptability to the environment (Goud et al., 2021).

The *ASIP* gene has been extensively studied in various cattle breeds and other animals, including French Brown Cattle (Royo et al., 2005), Australian Merino (Norris et al., 2008), Tibetan Sheep (Han et al., 2015), Chinese Simmental steers (Liu et al., 2019), and Nellore Cattle (Trigo et al., 2021). There has not been a study documenting the identification of the *ASIP* gene in Bali cattle. The current study aimed to explore the *ASIP* gene in Bali cattle and investigate its association with the occurrence of albinism and white-spotted coat color using direct sequencing.

MATERIALS AND METHODS

Ethical approval

The Animal Ethics Committee of Udayana University in Denpasar, Indonesia (Code ID: B/210/UN14.2.9/PT.01.04/2023) approved this study.

Study period and location

This study was conducted from June to November 2023. The study was conducted at several locations. Including Genetic analysis was carried out at the Molecular Genetics Laboratory, Division of Animal Breeding and Genetics, Department of Animal Production and Technology, Faculty of Animal Science, IPB University. Blood samples were collected from Bali cattle with standard (normal brown) and albino coat colors in Taro Village and the Livestock Center in the Sobangan Region, Bali Province.

Sample collection

A total of 68 blood samples from cattle were collected based on convenience and used in the current study, comprising standard Bali cattle (N = 39), Bali cattle with spotting (N = 9), and albino Bali cattle (N = 20; Figure 1). Samples of standard and white-spotted Bali cattle were collected from the Bali Cattle Breeding Centre, Jembrana (Bali Province), and Breeding Center Unit, Serading (West Nusa Tenggara). Albino cattle were sampled from Taro Village, Gianyar Regency, Bali, Indonesia. Approximately 5 mL of blood was obtained via jugular venipuncture using a venoject needle and Ethylenediaminetetraacetic acid (EDTA) vacutainer. The samples were stored at refrigeration temperature (-4°C) for a maximum of 3-4 days post-collection and subsequently processed for DNA extraction following Geneaid protocol (Geneaid Biotech Ltd., New Taipei City, Taiwan).

Figure 1. Standard coat color (**a**) and albino (**b**) in Bali cattle from the Bali Cattle, Indonesia.

Polymerase chain reaction amplification

To investigate the *ASIP* gene, primers were designed to cover exons 1–3 and the 3′UTR region (Table 1). Polymerase chain reaction (PCR) was carried out on an AB System thermocycler (Applied Biosystems, USA), manufactured in the United States of America. Each 25 µL reaction was prepared by combining 0.3 µL of both primers, 12.5 µL MyTaq HS RedMix, 9.9 µL of nuclease-free water, and 2 µL of DNA template. The cycling profile consisted of an initial denaturation step (95°C, 5 minutes), followed by 35 cycles of denaturation (95°C, 10 seconds), annealing (60°C, 20 seconds), and extension (72°C, 30 seconds), with a final elongation at 72°C for 5 minutes. Polymerase Chain Reaction (PCR) amplicons were analyzed by agarose gel electrophoresis, in which 3 µL of product was loaded on a 1.5% gel prepared with 0.5 × Tris-Borate-EDTA (TBE) buffer and stained with PeqGreen. Electrophoresis was performed at 100 V for approximately 30–35 minutes, and DNA fragments were visualized using UV transillumination. The sequencing of PCR products was performed using the Sanger sequencing method at the First Base Laboratory (Selangor, Malaysia) in collaboration with PT. Genetika Science, Indonesia.

Polymerase chain reaction products were distributed into 0.5 mL Eppendorf tubes with a volume of 5 µL. The PCR product was then combined with a mixture comprising 2 µL, consisting of 0.3 µL of the restriction enzyme, 0.7 µL of 10x buffer R, and 1 µL of nuclease-free water (NFW) in a 1.5 mL tube. Based on the results of the enzyme determination using NEB cutter, the restriction enzyme used was BspEl. The reaction mixture was incubated at 55°C for 2 hours. Following digestion with the restriction enzyme, DNA fragments were separated by electrophoresis on a 2% agarose gel at 100 V for 40 minutes, a standard condition for this fragment size range that produced clear bands without noticeable smearing, and visualized using a UV transilluminator in molecular biology labs, which is Bio-Rad (USA). The observed banding patterns were compared with a molecular size marker to estimate the fragment length, and genotypes were identified based on the resulting band sizes.

Table 1. Nucleotide sequences used as primers in PCR for the identification of *ASIP* genes

***ASIP* Gene Targeted**	**Primer sequence (5' to 3')**	**Amplicon Length (bp)**	**Annealing Temperature (°C)**	**Reference**
Exon-1	F:5'-TCAGAGTACCAGCCCAAAGA-3'			
	R:5'-TATCGGCTTGGGGAGTGTTT-3'	355		
Exon-2	F:5'-TTCATCCTCTCCCCAACCCT-3'		60	Manual design using Primer3 and PrimerStat websites
	R:5'-TCAGTGCTTGAGGTCAGGAC-3'	317		
Exon-3	F:5'-GAGTGGGGAGGACGTAGATG-3'			
	R:5'-GGGACTAGGCGAAGGGAAAA-3'	462		

F: Forward, R: Reverse, PCR: Polymerase chain reaction, *ASIP: Agouti Signaling Protein* gene.

Sequencing single-nucleotide polymorphisms identification and statistical analysis

The *ASIP* gene sequences were analyzed using FinchTV and BioEdit version 7.2 (Hall, 1999). Single-nucleotide polymorphisms (SNPs) were detected by aligning the sequences with Clustal W implemented in MEGA version 10 (Tamura et al., 2013). Population genetic parameters, including genotype and allele frequencies, observed and expected heterozygosity, and Hardy–Weinberg equilibrium (HWE), were calculated using POPGEN version 1.32 (Yeh et al., 2000). Hardy-Weinberg equilibrium (HWE) was analyzed using the chi-square test (2) to determine the HWE value in a population. The Hardy–Weinberg equilibrium test showed a $p > 0.05$, indicating no significant deviation from equilibrium, and $p \leq 0.05$ indicating significance.

RESULTS AND DISCUSSION

The coding regions (exon-1, exon-2, and exon-3) of the *ASIP* gene were successfully amplified with consecutive PCR product lengths of 355 bp, 317 bp, and 462 bp at an annealing temperature of 60°C, as shown in Figure 2. The success of the amplification process is crucial for obtaining reliable sequencing results. Several factors determining the success of gene amplification can be attributed to the design of the primers used (Housley et al., 2006). A well-designed primer can be identified by the percentage of guanine and cytosine content, typically ranging between 50% and 60%, a melting temperature (50-65°C), and a temperature difference between the forward and reverse primers of less than 5°C (Siswanto et al., 2022).

Figure 2 illustrates the sequence of the coding region of the *ASIP* gene, which was successfully sequenced and aligned. The alignment results revealed that the *ASIP* gene's exon-1 and exon-2 regions exhibited monomorphic characteristics, whereas exon-3 in Bali cattle displayed polymorphism or diversity. However, the diversity in the *ASIP* gene cannot be further analyzed to determine coat color markers in Bali cattle. This is due to the non-specific nature of the identified SNP or mutations, preventing the differentiation of standard, albino, and white-spotted Bali cattle coat

colors (Jakaria et al., 2023). Analysis of exon-3 of the *ASIP* gene revealed polymorphisms that did not correlate with coat color in Bali cattle. This lack of correlation may be due to the presence of silent (synonymous) mutations, which do not alter the amino acid sequence of the protein and are thus unlikely to affect its function (Girardot et al., 2006; Royo et al., 2005). Nevertheless, a novel aspect of this study is the discovery of a new SNP, g. 498 A > G was exclusively found in Bali cattle sequences. Consequently, this newly identified SNP has the potential to serve as a genetic marker, particularly for the coat color of Bali cattle. The g.498 A > G SNP in exon-3 of the *ASIP* gene has been implicated in coat color variation by modulating *ASIP* protein function (Zhang et al., 2017). This alteration affects melanogenesis pathways, potentially influencing pigmentation (Guo et al., 2019; Lei et al., 2021). These genetic variations are critical for understanding the molecular mechanisms underlying coat color diversity in mammals.

Figure 4 depicts the results of translating nucleotide base sequences into amino acids in the Bali cattle. The involvement of *ASIP* with other genes that control coat color is shown in Figure 5. The *ASIP* gene plays a significant role in regulating coat color formation. The alignment results of the amino acids in Bali cattle and other cattle breeds are shown in Figure 4. Figure 4 indicates the differences in the amino acids of valine and serine in Bali cattle compared to the Bos mutus grunniens breed. The uniformity of a livestock population is influenced by several factors, including inbreeding (Visscher, 2001), selection, migration, and genetic drift (Yeaman and Whitlock, 2011). Table 1 shows the genotype and allele frequency results for Bali cattle. The highest genotype frequency was observed in white-spotted Bali cattle with the GG genotype (0.67), making allele G the most prevalent in white-spotted Bali cattle. Additionally, in the population diversity of Bali cattle analyzed, white-spotted Bali cattle did not have the genotype AA (0.00), resulting in the lowest allele frequency of A compared to other herds.

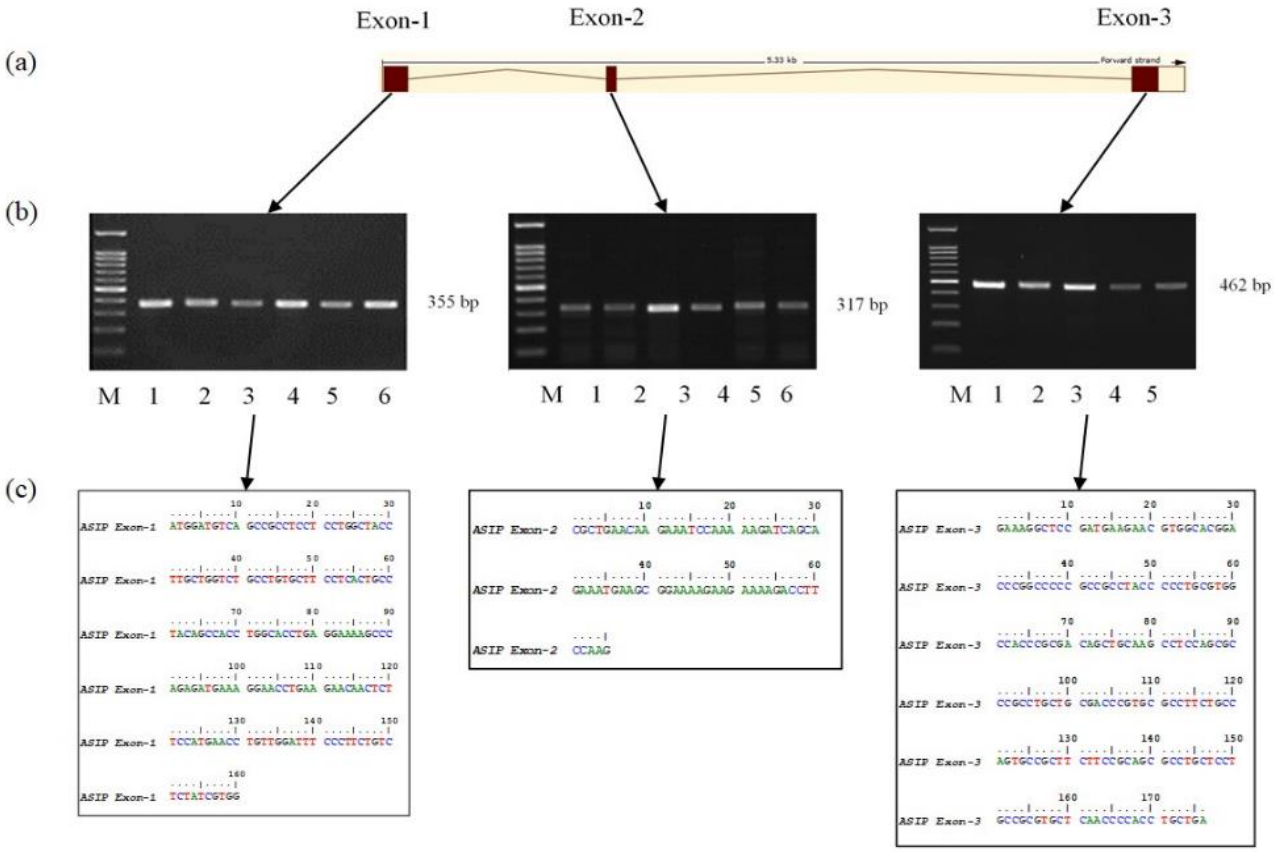


Figure 2. *ASIP* gene structure (**a**), PCR product (**b**), and coding region sequence of the *ASIP* Gene (**c**) in Bali Cattle, Indonesia.

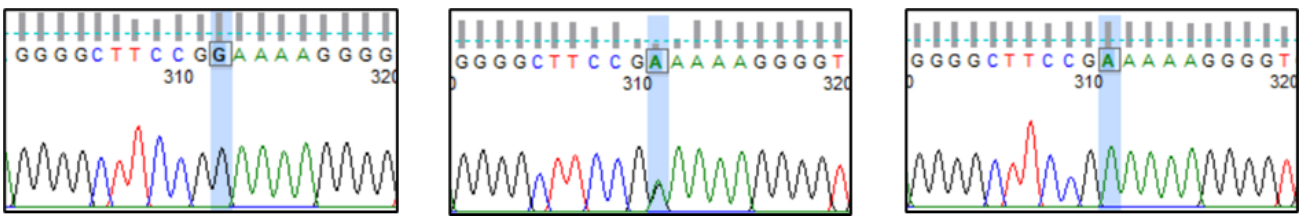
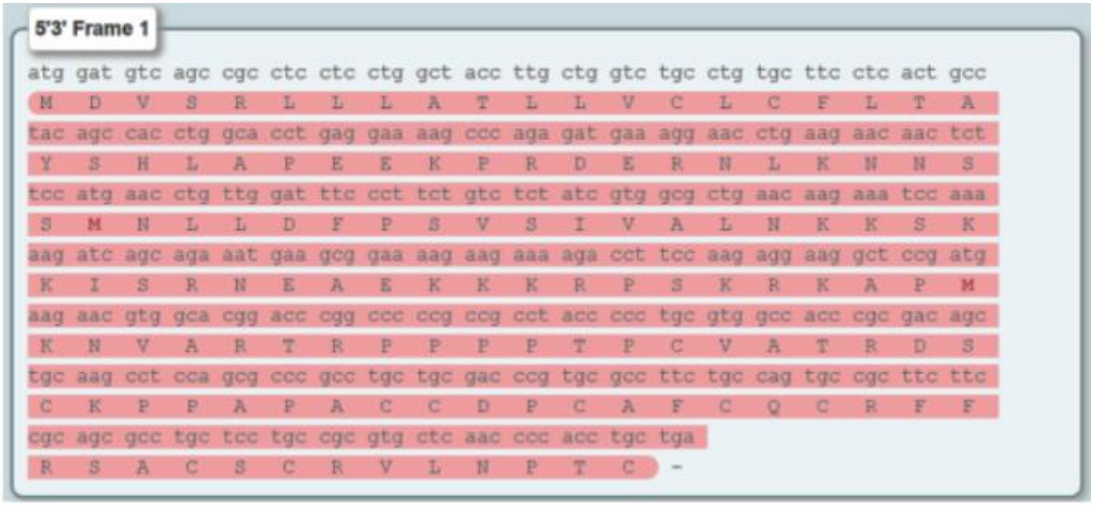


Figure 3. New SNP g.498 A > G in the exon-3 3'UTR region of the *ASIP* gene in Bali cattle, Indonesia.

(a)



(b)

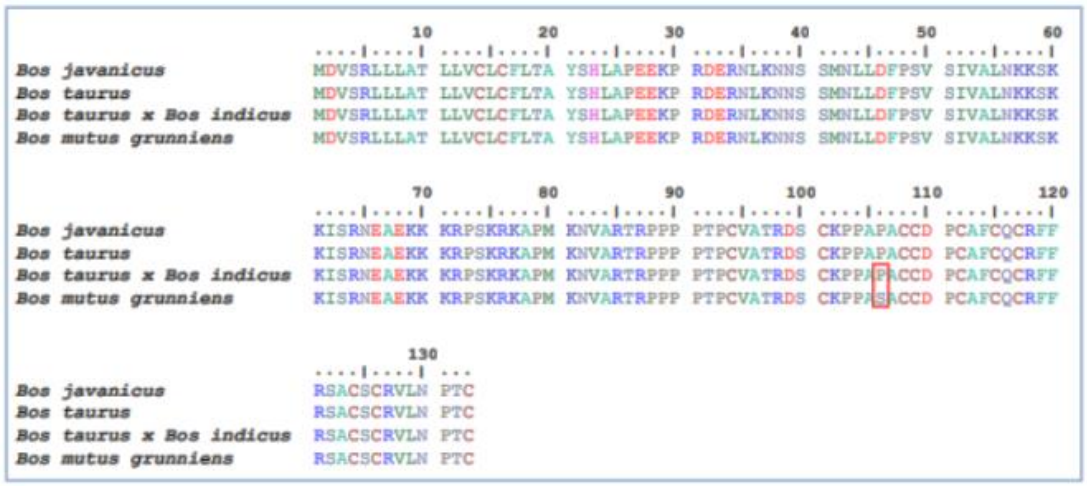


Figure 4. The nucleotide sequence of the *ASIP* gene was translated into amino acids with Expasy (**a**), and amino acid alignment of Bali cattle (*Bos javanicus*) against other breeds was conducted using MEGA (**b**).

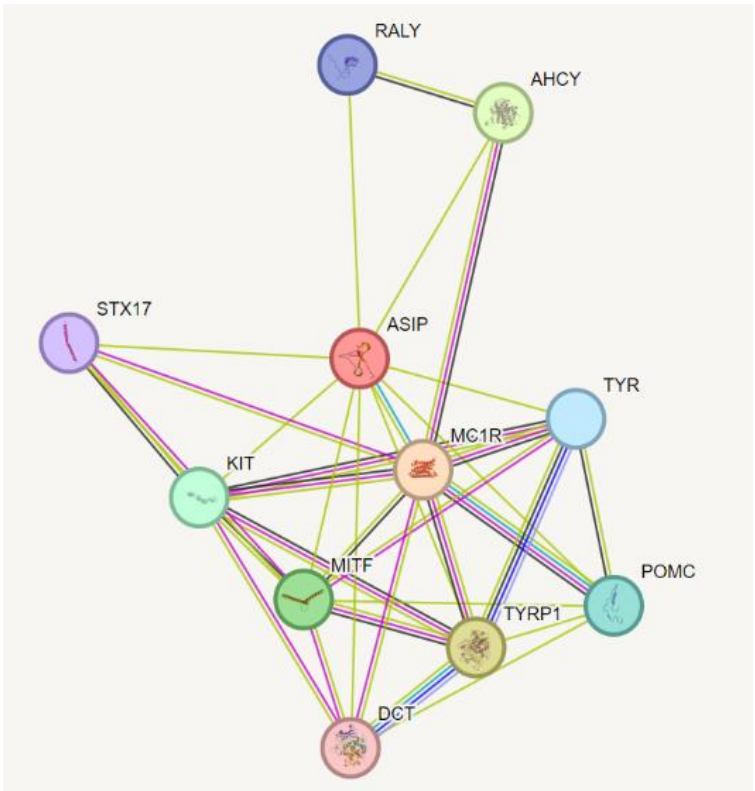


Figure 5. Involvement of the *ASIP* gene and other genes in controlling coat color in cattle (Source: www.string-db.org/).

Table 2 shows that all populations of standard, albino, and white-spotted Bali cattle have non-significant ($p > 0.05$), indicating that all these Bali cattle populations are in the Hardy-Weinberg equilibrium status. Although all Bali cattle populations (standard, albino, and white-spotted) showed non-significant χ^2 values ($p > 0.05$), these results should be interpreted with caution. Non-significant χ^2 values do not necessarily indicate Hardy–Weinberg equilibrium (HWE), particularly when statistical power is low. Bosco et al. (2012) showed that χ^2 tests may fail to detect deviations from HWE in randomly mating populations with low power, leading to false negatives, whereas non-random mating populations may produce false positives. Furthermore, genotyping errors can reduce the power of χ^2 tests, especially with rare allele frequencies (Wang and Shete, 2012). Therefore, conclusions regarding equilibrium status should consider both statistical power and data quality rather than relying solely on non-significant χ^2 values. Although all Bali cattle populations (standard, albino, and white-spotted) showed non-significant χ^2 values ($p > 0.05$), these findings should be interpreted carefully. A non-significant result does not automatically indicate Hardy–Weinberg equilibrium, particularly under conditions of low statistical power, such as small sample sizes or limited genetic diversity.

Table 2. Evaluation of SNP g.498 A > G in Bali cattle by RFLP-PCR

Phenotype	Number	Genotype frequency			Allele frequency		H_o	H_e	χ^2 test
		GG	AA	GA	A	G			
Albino	20	0.45	0.10	0.45	0.32	0.68	0.45	0.44	Ns
Standard	36	0.44	0.17	0.39	0.36	0.64	0.39	0.47	Ns
White-spotted	9	0.67	0.00	0.33	0.17	0.83	0.33	0.29	Ns

SNP: Single nucleotide polymorphism, N: Simple size, H_o: Observed heterozygosity, H_e: Expected heterozygosity, RFLP-PCR: Restriction fragment length polymorphism polymerase chain reaction Ns: Not significant ($p > 0.05$)

A study related to the *ASIP* gene in cattle has been previously conducted, which reported that the coding region of the *ASIP* gene in Spanish and French Brown cattle did not significantly influence the coat color formation process (Royo et al., 2005). Similarly, while exon-1 and exon-2 of the *ASIP* gene were monomorphic in Bali cattle, this observation is consistent with the findings in Brown cattle, suggesting that these regions have little effect on coat color (Royo et al., 2005). A polymorphism in intron 2 of the *ASIP* gene was observed in Hanwoo cattle (Suk Kim et al., 2007); however, it lacked conservation in the population. In addition to regulating coat color, the *ASIP* gene has other general roles, such as controlling the milk metabolism process, as reported by Xie et al. (2022). A study has been conducted on other livestock breeds, such as the Modense breed (Bertolini et al., 2022). However, no results have been reported on the *ASIP* gene and its relationship with coat color (standard, albino, and white-spotted) in Bali cattle.

CONCLUSION

Analysis of the *ASIP* gene showed that the exon-1 and exon-2 regions were monomorphic, indicating uniformity among Bali cattle. In contrast, exon-3 displayed polymorphism, and a novel SNP (g.498 A > G) was identified, suggesting potential genetic variation that may contribute to coat color diversity in this breed. This newly discovered SNP is unique to Bali cattle. However, it was non-specific, and a mutation in the *ASIP* gene was identified in albino, standard, and white-spotted Bali cattle. Consequently, this SNP cannot be considered a candidate genetic marker for coat color in Bali cattle, particularly in albino and white-spotted individuals. Amino acid alignment results of Bali cattle (*Bos javanicus*) with other cattle breeds showed consistent outcomes. Future studies should focus on exploring other candidate genes, regulatory regions, and gene–environment interactions to better understand the genetic basis of coat color variation in Bali cattle. Additionally, expanding the sample size and applying whole-genome sequencing approaches may provide more comprehensive insights into the molecular mechanisms underlying albinism and white-spotted phenotypes.

DECLARATIONS

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Availability of data and materials

All data and information analyzed in this review were obtained from publicly accessible articles that are properly cited in the manuscript. More information is available upon reasonable request from the corresponding author.

Authors' contributions

Kholijah Kholijah, Sri Darwati, Muhammad Fakhrul Ulum, Ronny Rachman Noor, Jakaria Jakaria. Kholijah Kholijah and Jakaria Jakaria collected the data and provided the original draft of the manuscript, while Sri Darwati, Muhammad Fakhrul Ulum, Ronny Rachman Noor, and Jakaria Jakaria reviewed it. All authors considered and agreed on the final version of the manuscript for publication in this journal.

Ethical considerations

All authors have checked for ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy. The authors used ChatGPT (OpenAI) and Grammarly solely for language editing purposes; no AI tools were used for data analysis or manuscript content generation.

Competing interests

The authors declare no conflicts of interest.

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