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Detection of Genetic Diversity of Sambar Deer (*Cervus unicolor*) Using Inter Simple Sequence Repeat Molecular Markers

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ABSTRACT

The Sambar deer (*Cervus unicolor*) is a species facing the threat of population decline due to poaching. This study aims to analyze the genetic diversity of Sambar Deer from East Kalimantan using the Inter Simple Sequence Repeat (ISSR) molecular marker with primers P01 and P02. There were 10 sambar deer, eight females and two males, from Pembibitan Ternak dan Hijuan Pakan Ternak, Penajam Paser Utara, East Kalimantan. Blood samples were obtained from the jugular vein using an EDTA project tube. The samples were processed through Polymerase Chain Reaction (PCR) and electrophoresis to detect polymorphism patterns. This research showed a DNA average purity of 1.382 and an average concentration of 13.205; primer P01 produced three haplotype diversity, and primer P02 produced two haplotype diversity. The Polymorphic Information Content (PIC) values were 0.632 for primer P01 and 0.304 for primer P02. Dendrogram analysis revealed three population clusters based on genetic diversity. The conclusion indicates polymorphism, with primer P01 showing higher polymorphism than primer P02.

Keywords: Genetic diversity; ISSR; Polymorphism; DNA; *Cervus unicolor*.

INTRODUCTION

Ecological diversity encompasses ecosystem, species, and genetic diversity, which play critical roles in maintaining ecological balance (Sutoyo, 2010). The sambar deer, a significant germplasm resource, is currently under threat due to pressures such as poaching, leading to a decline in its population. To address this, activities such as exploration, inventory, evaluation, and conservation are essential for enriching and preserving germplasm, thereby supporting the development of superior genetic varieties (Sutoyo, 2010). These efforts are crucial for safeguarding invaluable and irreplaceable natural resources (M. R. Mohammadabadi, 2017). Species with limited genetic diversity exhibit reduced adaptability to environmental changes, as a population's resilience to such changes is directly linked to its genetic variability (M. Mohammadabadi et al., 2017). Consequently, conserving biodiversity is vital to minimizing the loss of genetic variation. As a local species with potential for development, the sambar deer can benefit from genetic quality improvement initiatives that focus on maintaining its genetic purity and ensuring its long-term preservation.

Sambar deer from East Kalimantan have relatively high genetic diversity compared to Sambar deer from South Sumatra; this is indicated by the haplotype index Hd= 1,000 for the East Kalimantan population and Hd = 0.9545 for the South Sumatra population. (Wirdateti, 2012). The wide distribution of Sambar deer from South Asia to the islands of Sumatra and Kalimantan is one factor that influences the population structure and high genetic diversity of Sambar deer. (Wirdateti, 2012). The lack of information about the biodiversity of flora and fauna raises concerns about the extinction of species that have not been recognized and are needed for conservation and utilization. Inter Simple Sequence Repeat is one of the fast and affordable molecular markers; ISSR can detect high polymorphism and variation between populations both geographically separated and within one population. Identification of sambar deer is one of the important things to know about kinship relationships between each other, to avoid inbreeding, and to know the status of sambar deer offspring. (Puspa Pratiwi, 2014). ISSR in animal husbandry was previously used to determine the DNA fingerprints of three goat breeds in Nigeria. (Etta, 2023). The ISSR method has been used in genetic diversity studies in several species. (M. Mohammadabadi et al., 2017) such as cattle (Ghasemi et al., 2010), cattle, goat, and sheep (Askari et al., 2011), fish (Zhigileva et al., 2013), mouse (Renny et al., 2014).

ISSR (Inter Simple Sequence Repeat) markers have significant potential in identifying

genes associated with important traits. While these markers have been widely used in plant genetic diversity studies, their application in animal research, particularly about breeding value associations, remains limited. Research on ISSR markers associated with selected traits in animal breeding is crucial for improving breeding efficiency. This is particularly true for species like the sambar deer, which holds both economic value and conservation relevance. The use of ISSR markers can aid in identifying genetic variants that influence quality or productivity. Therefore, this study aims to explore and identify ISSR markers in sambar deer to open opportunities for enhancing breeding programs and gaining deeper insights into the inheritance patterns of specific traits in this species.

MATERIALS AND METHODS

The current study used material, namely blood samples of sambar deer (*Cervus unicolor*). 10 sambar deer consisting of 8 females and two males from the (UPTD) Pembibitan Ternak dan Hijuan Pakan Ternak, Penajam Paser Utara, East Kalimantan. In this study, the initial stage used was the preparation stage. Sampling was done by collecting 3 µL of blood through the jugular vein using a projecting tube containing EDTA. The DNA extraction was done using the Genome Extraction Kit (Geniad, Taiwan). PCR reactions were made up to 10 µL; the primer used was primer P01 with oligonucleotide base sequence 5'-AGA GAG AGA GAG AGA GAG -3' and primer P02 with oligonucleotide base sequence and primer P02 with oligonucleotide base sequence and primer P02 with oligonucleotide base sequence and primer P02 with oligonucleotide base sequence 5' – GAG AGA GAG AGA GAG AGA -3'(M. R. Mohammadabadi, 2017) (M. Mohammadabadi et al., 2017). PCR program used pre-denaturation 94°C for two minutes, denaturation 94°C for one minute thirty seconds, annealing 52,5°C for the use of primer P01 while for primer P02 using temperature annealing 59,0°C for one minute, extension 72°C for one minute, final extension 72°C for five minutes, and cooling down 4°C for five minutes, cycles used in the PCR program are thirty-five. Electrophoresis was carried out with 2% agarose gel at 100V for 60 minutes. (Destomo et al., 2024), then visualized at UV Gel Documentation Uvitec Platinum V10 under ultraviolet light (Babii et al., 2016). Data analysis was carried out based on variations in DNA fragments seen in each sample, and the parameters used included the number of fragments, Shanon index, and Polymorphic Information Content (PIC). Data were analyzed using mathematical formulas to see the level of genetic diversity of the animals studied:

ne: $1 / \Sigma Pij2$

I : $- \Sigma Pi \ln (Pi)$

PIC : $1 - \Sigma pi2$

Description: ne is the number of effective alleles, I am Shanon index, PIC is total polymorphic fragments (Weir, B.S., 1990; Putra et al., 2024).

RESULTS AND DISCUSSION

DNA Purity and Concentration

The purity value and concentration of Sambar deer DNA produced from the study with an average purity of 1.382 ng/µL and an average concentration of 13.205 ng/µL have been presented in Table 1.

Table 1. Purity and concentration test result of Sambar deer

Parameters	Average (ng/ µL)	SD	Maximum	Minimum
Purity	1.382	0.141	1.629	1.175
Concentration	13.205	5.838	24.350	6.900

Description: Sambar deer DNA is contaminated, as seen from the results of the purity value produced below 1.8 - 2.0.

The purity value and concentration of sambar deer DNA produced from the study had an average value of 1.382 ng/µL and an average concentration of 13.205 ng/µL. The purity of Sambar deer DNA has experienced contamination; this is indicated by the average purity of DNA produced of 1.382 ng/µL (Table 1.) is below 1.8. The level of DNA purity can be seen if the A260/A280 ratio ranges from 1.8 - 2.0 (Lucena-Aguilar et al., 2016). DNA purity values less than 1.8 can be indicated due to other contaminants such as proteins or others, while when the purity value exceeds 2.0, it can indicate contamination by RNA (Wardana & Muslih, 2021). Some of the most common causes are contamination in the sample and contamination in the isolation process (Wardana & Muslih, 2021). Previous studies using the same kit and multiple

methods revealed that method 1 had an average concentration of 5.99 with a purity of 36.21, method 2 had a concentration of 6.33 with a purity of 9.22, and method 3 had a concentration of 17.54 with a purity of 2.84 (Pratama & Arisuryanti, 2022). The concentration test yielded 6.900-24.350, which is still a relatively low absorbance ratio; nevertheless, the extraction findings can be verified with a PCR tool, allowing DNA's concentration and purity value to be determined by the sensitivity of the PCR tool. Several earlier researches using non-invasive blood collection have demonstrated poor DNA quality and purity (Ando et al., 2020).

PCR-ISSR on Sambar Deer (*Cervus unicolor*)

The PCR-ISSR amplification profiles generated in this study using primers P01 and P02 are illustrated in Figures 1 and 2, respectively. Optimal annealing temperatures, ranging from 45°C to 62°C, were determined through a systematic temperature gradient optimization protocol before final amplification. This gradient-based approach ensured precise alignment of primer-template hybridization specificity, a critical factor in minimizing non-specific binding and enhancing amplification efficiency. The thermocycling parameters, including Taq polymerase concentration, dNTP stoichiometry, and $MgCl_2$ concentration, were standardized to maintain reaction fidelity. Electrophoretic analysis of the amplicons revealed distinct banding patterns, with primer P01 producing polymorphic fragments spanning 200–1500 base pairs (bp), while P02 yielded bands within 300–1200 bp, as resolved on 1.5% agarose gels stained with ethidium bromide—the reproducibility of protocols in ISSR-based genotyping.

From a molecular, these profiles were validated through triplicate runs, with molecular weight markers (1 kb ladder) serving as reference standards. Notably, the observed variability in band intensity across lanes correlates with template DNA quality and primer annealing kinetics, underscoring the necessity of a stringent optimization biology perspective; the temperature gradient optimization step was pivotal in identifying the narrow thermal window (48–52°C for P01; 50–55°C for P02) that maximized primer specificity while mitigating secondary structure interference. This aligns with established principles of PCR stringency, where Tm (melting temperature) adjustments directly influence primer-template duplex stability. The resultant amplification efficiency and band clarity (Figures 1, 2) confirm the robustness of ISSR markers for detecting interspersed repetitive sequences in sambar deer genomic DNA. Such methodological precision is indispensable for downstream applications, including population genetic analyses, marker-trait association studies, and conservation-oriented genetic resource management. These findings underscore the utility of ISSR markers in elucidating microsatellite-associated genomic variations, thereby advancing molecular breeding strategies in non-model species.

The electrophoretic profiles in Figures 1 and 2 reveal distinct DNA banding patterns, with primer P01 amplifying fragments ranging from 350–900 base pairs (bp) and primer P02 generating bands between 300–750 bp. These results align with previous studies demonstrating species-specific ISSR polymorphism ranges. For instance, Balinese cattle (*Bos javanicus*) exhibited polymorphic loci within 550–1000 bp using the $(G)_9C$ ISSR marker, revealing three haplotype variations: H1 (550 bp), H2 (1000 and 550 bp), and H3 (1000, 700, and 550 bp) (Putra et al., 2024).

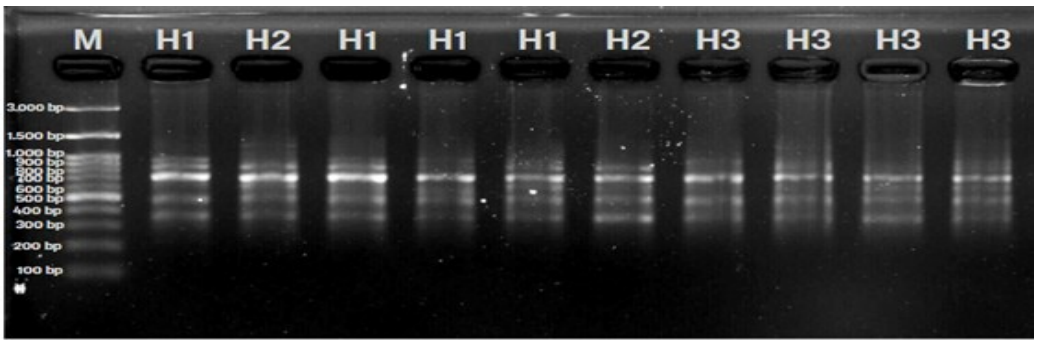

Figure 1. Amplification results of primer P01 Sambar deer using annealing temperature 52.05°C

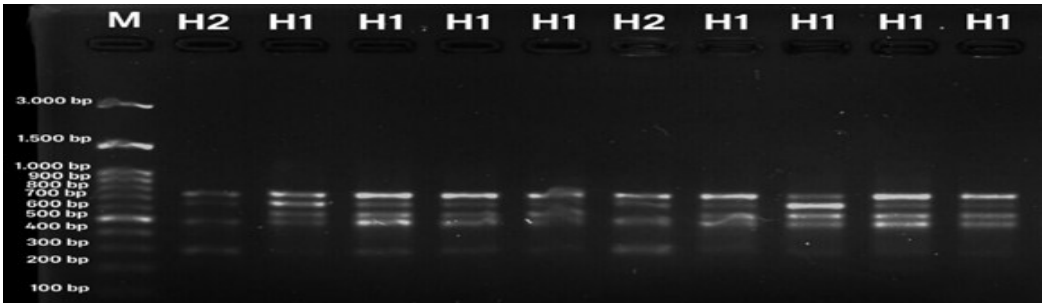

Figure 2. Amplification results of primer P02 Sambar deer using annaeling temperature of 59.0°C

Similarly, short fat-tailed sheep (*Ovis aries*) in South Tuva, Siberia, displayed ISSR-PCR fragments spanning 160–1750 bp (Kol et al., 2010), while Bulgarian goat breeds (*Capra hircus*) produced 19 polymorphic bands, underscoring the utility of ISSR markers in detecting genetic diversity (Kostova & Bojinov, 2018).

This study observed variable DNA banding patterns across all sambar deer (*Cervus unicolor*) samples, with primer P01 demonstrating higher polymorphism than P02. For example, in Bulgarian goats, primer-specific differences yielded varying numbers of informative polymorphic bands, highlighting the need for empirical primer selection in population genetics. The differential polymorphism levels between primers P01 and P02 may be attributed to primer binding site distribution variations across the sambar deer genome. ISSR markers amplify inter-simple sequence repeat regions and are highly sensitive to primer design and annealing conditions. Primer P01's broader polymorphism range (350–900 bp) suggests it targets more variable microsatellite-rich regions, whereas P02's narrower range (300–750 bp) may reflect conserved genomic regions or suboptimal primer-template compatibility. These observations align with molecular principles governing ISSR amplification, where primer length, GC content, and annealing temperature influence hybridization specificity and fragment diversity (Zietkiewicz et al., 1994).

Faint bands in specific lanes (Figures 1, 2) may indicate low-copy-number DNA regions, heterozygosity, or stochastic amplification artifacts, necessitating stringent optimization of Mg^{2+} concentration, Taq polymerase fidelity, and thermal cycling parameters. Such methodological rigor is critical for minimizing technical noise and enhancing the reliability of ISSR data in conservation genetics. From a molecular ecology perspective, the higher polymorphism detected by primer P01 in sambar deer underscores its utility for population structure analyses, inbreeding assessments, and marker-assisted conservation strategies. The observed haplotype-like variations in Balinese cattle (Putra et al., 2024) and fragment size disparities across species further validate ISSR's applicability in comparative genomics. However, the limited polymorphism of P02 emphasizes the need for multi-primer approaches to comprehensively assess genetic diversity, as single primers may overlook critical loci.

The variability in ISSR banding patterns reflects underlying allelic diversity, a key determinant of population resilience. Species with reduced genetic diversity, as inferred from limited polymorphic bands, face heightened extinction risks due to diminished adaptive potential (Frankham et al., 2017). For sambar deer, a species of ecological and economic significance, these findings provide a genomic toolkit for monitoring genetic health, identifying unique haplotypes, and designing targeted breeding programs. Future studies should integrate ISSR data with mitochondrial or nuclear markers to resolve phylogenetic uncertainties and enhance conservation prioritization.

Genetic Diversity

The analysis of ISSR markers in sambar deer (*Cervus unicolor*) revealed distinct patterns of genetic diversity, as evidenced by the haplotype distributions and fragment sizes generated by primers P01 and P02. Primer P01 amplified three haplotypes (H1, H2, H3), each characterized by unique fragment profiles (Table 2). Haplotype H1, observed in 40% of the samples (4/10), produced five fragments (900, 800, 700, 500, and 350 bp), indicating a relatively high level of allelic richness. In contrast, H3, also present in 40% of samples, exhibited fewer fragments (700, 500, 350 bp), suggesting a potential loss of more significant repeat regions, possibly due to mutational erosion or selective pressures (Pérez-Espona et al., 2008). The intermediate haplotype

H2 (800, 700, 500, 350 bp), found in only 20% of samples (2/10), represents a rare genetic subgroup, highlighting the presence of minor lineages within the population. Primer P02, meanwhile, generated two haplotypes: H1 (750, 650, 550, 500, 300 bp) dominated 80% of samples (8/10), while H2 (750, 650, 300 bp) was limited to 20% (2/10). This skewed distribution toward H1 in P02 suggests reduced allelic diversity at these loci, potentially reflecting genetic drift or historical bottlenecks that have homogenized this genomic region (Frankham et al., 2017).

The haplotype diversity indices further quantify these patterns. For P01, the total diversity value of 0.36 reflects moderate heterogeneity, driven by the balanced frequencies of H1 and H3. In contrast, P02's higher diversity index (0.68) arises from the pronounced dominance of H1, which paradoxically masks lower actual allelic variation due to the rarity of H2. These results align with the findings of Kostova and Bojinov (2018), who demonstrated that primer specificity significantly influences the detection of polymorphic loci. The broader polymorphism range of P01 (350–900 bp) likely targets more variable microsatellite regions, as described in genome fingerprinting studies (Zietkiewicz et al., 1994), while P02's narrower range (300–750 bp) may anneal to conserved or less diverse loci. The presence of shared smaller fragments across haplotypes (e.g., 500 and 350 bp in P01; 300 bp in P02) implies conserved sequences that may play critical functional roles, whereas variable larger fragments (e.g., 900 bp in P01-H1; 750 bp in P02-H1) likely correspond to polymorphic repeat regions associated with adaptive traits (Reddy et al., 2002).

From a conservation perspective, the coexistence of multiple haplotypes signifies residual genetic variation, which is vital for the population's resilience to environmental changes (Allendorf et al., 2013). However, the rarity of H2 in both primers raises concerns about genetic erosion, as small populations are prone to losing rare alleles through drift or inbreeding (Frankham et al., 2017). The loss of larger fragments in H3 (P01) and the absence of intermediate fragments in H2 (P02) may reflect genomic instability or selective sweeps, processes that reduce diversity over time. These findings align with studies in other species, such as Balinese cattle (Putra et al., 2024) and Bulgarian goats (Kostova & Bojinov, 2018), where ISSR markers similarly revealed haplotype variations linked to adaptive potential and population bottlenecks.

Table 2. Genetic Haplotype Diversity of Sambar Deer Using ISSR Markers

Marker	Haplotype	Number of Locus	Size* (bp)	Frequency (N)		Total
				Frequency	Frequency^2	
P01	1	5	900; 800; 700; 500; 350	0.4 (4)	0.16	0.36
	2	4	800; 700; 500; 350	0.2 (2)	0.04	
	3	3	700; 500; 350	0.4 (4)	0.16	
P02	1	5	750; 650; 550; 500; 300	0.8 (8)	0.64	0.68
	2	3	750; 650;300	0.2 (2)	0.04	

Table 3. Genetic Diversity of Sambar Deer Using ISSR Markers

Marker	n_e	I	PIC
P01	1.054	2.777	0.64
P02	5.504	1.470	0.32

The phenomenon observed in Table 3 pertains to the genetic diversity of Sambar deer (*Cervus unicolor*) as assessed using Inter-Simple Sequence Repeat (ISSR) markers. ISSR markers are widely used to evaluate genetic diversity by amplifying regions between microsatellites, providing insights into the level of genetic variation within a population. The table presents three key metrics: effective number of alleles (ne), Shannon's information index (I), and Polymorphism Information Content (PIC). Primer P01 yielded values of ne = 1.054, I = 2.777, and PIC = 0.64, while primer P02 produced ne = 5.504, I = 1.470, and PIC = 0.32. The higher value for primer P02 suggests that it amplifies more alleles or fragments, indicating more polymorphic

loci in the target regions. However, the lower I and PIC values for primer P02 imply that the alleles it amplifies are less informative or unevenly distributed in terms of frequency, possibly due to the presence of rare alleles or skewed allele frequencies in the population (Kumar et al., 2021). This discrepancy highlights the influence of primer selection on genetic diversity assessments, as different primers may target genomic regions with varying levels of polymorphism and informativeness (Wang et al., 2020).

Compared to other studies, the genetic diversity of Sambar deer appears higher than that of short fat-tailed sheep in southern Tuvan Siberia, which reported values of I = 0.063, ne = 1.081, and PIC = 0.454 (Kol et al., 2010). However, the PIC values for Sambar deer in this study are lower than those reported for Polar Deer (*Rangifer tarandus L.*), where ISSR markers (AG)9C and (GA)9C yielded PIC values of 0.7307 and 0.7488, respectively (Kol & Lazebny, 2006). These comparisons underscore the variability in genetic diversity across species and the importance of marker selection in obtaining accurate assessments. Recent studies, such as those by Kumar et al. (2021) and Wang et al. (2020), emphasize the need to use multiple markers and primers to capture a comprehensive picture of genetic diversity, as different primers may amplify regions with varying levels of polymorphism and informativeness. In conclusion, the observed differences in genetic diversity metrics between primers P01 and P02 reflect the variability in the genomic regions they amplify, highlighting the importance of employing a combination of markers to ensure robust and reliable genetic diversity assessments.

Dendrogram of Sambar Deer (*Cervus unicolor*) Based on PCR-ISSR.

The phenomenon of "the length of fragments to be the same cluster" refers to grouping individuals into clusters based on the similarity of their genetic fragments, as revealed by PCR-ISSR analysis. In this study, the dendrogram constructed from the PCR-ISSR data of Sambar deer (*Cervus unicolor*) using primers P01 and P02 revealed the emergence of three distinct clusters. This clustering is based on the principle that individuals with similar fragment lengths reflecting shared genetic characteristics are grouped, indicating their genetic relatedness. The level of genetic diversity and kinship relationships within the population influences the formation of these clusters. Specifically, the dendrogram showed that four samples (40%) were categorized in Cluster 1, 4 samples (40%) in Cluster 2, and 2 samples (20%) in Cluster 3. This distribution suggests a moderate level of genetic diversity in the Sambar deer population, as the individuals are not genetically identical but can be grouped into distinct clusters based on their genetic profiles (Febrita & Amaniyah, 2022).

Comparisons with other studies highlight the variability in genetic diversity across species. For instance, Destomo et al. (2024) found only 2 clusters in Saburai goats using the same ISSR primers, indicating lower genetic diversity than the Sambar deer in this study. Similarly, Askari et al. (2011) reported varying numbers of clusters in different species, with goats forming 4 clusters, sheep forming 3 clusters, local cattle from Bam (Iran) forming 5 clusters, and Holstein cattle forming 4 clusters. These differences underscore the influence of species-specific genetic diversity and population structure on cluster formation. The higher number of clusters in some species, such as cattle, suggests greater genetic diversity, while fewer clusters, as seen in Saburai goats, may indicate lower diversity or higher levels of inbreeding (Askari et al., 2011; Destomo et al., 2024).

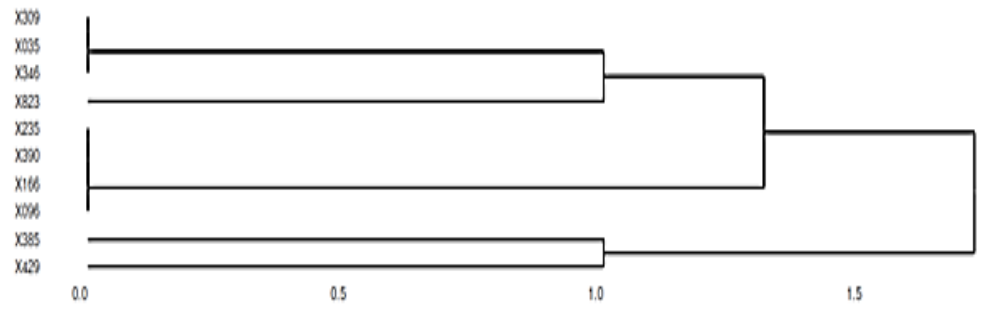

Figure 3. Dendrogram of Sambar Deer Based on PCR-ISSR Using Primer P01 and P02.

Several factors, including mating patterns, inbreeding levels, and evolutionary history, influence the formation of clusters in a population. Random mating maintains genetic diversity, while non-random mating, such as inbreeding, can reduce diversity and lead to fewer clusters. Additionally, population bottlenecks, founder effects, and gene flow can shape the genetic structure of a population, further influencing cluster formation (Febrita & Amaniyah, 2022). In the case of the Sambar deer, the emergence of three clusters reflects a balanced genetic structure, likely influenced by natural mating patterns and moderate levels of genetic diversity.

In conclusion, clustering Sambar deer into three groups based on fragment length similarity highlights the utility of ISSR markers in assessing genetic diversity and population structure. Compared to other species, the moderate level of genetic diversity observed in this study provides valuable insights into the genetic health and evolutionary dynamics of the Sambar deer population. These findings are crucial for informing conservation and management strategies, ensuring the preservation of genetic diversity in this species.

CONCLUSION

Applying ISSR markers with primers P01 and P02 in Sambar deer from East Kalimantan demonstrated polymorphism, with primer P01 exhibiting greater genetic diversity than primer P02. Primer P01 generated three haplotype variations, whereas primer P02 produced only 2, suggesting that P01 amplifies more genetically variable genome regions. This variation underscores the significance of primer selection in genetic analyses, as different primers may target genomic regions with differing levels of polymorphism. The higher genetic diversity identified by P01 indicates that the Sambar deer population possesses a strong genetic foundation, enhancing its resilience to environmental changes and disease. Conversely, the lower diversity observed with P02 emphasizes the necessity of employing multiple primers to evaluate genetic variation thoroughly. These results are vital for conservation efforts, as insights into genetic diversity and population structure inform strategies to preserve genetic integrity, mitigate inbreeding, and support the species' long-term viability.

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