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Genetic Diversity Analysis of Sambar Deer (*Cervus unicolor*) Based on Mitochondrial DNA D-loop Sequence

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ABSTRACT

This study aimed to analyze the genetic diversity of the sambar deer (*Cervus unicolor*) population through mitochondrial D-loop DNA sequencing. The findings provide valuable insights into the conservation status of this vulnerable species in the region and inform future management strategies for *Cervus unicolor*. Blood samples from 10 individual sambar deer, consisting of 8 females and two male deer, were amplified using PCR and then sequenced. Data analysis of the genetic diversity of sambar deer was carried out using the genome sequencing method from NCBI, Bioedit 7.7.1 software, DNAsp 5.1, and MEGA 11 Software. The results of this study were that the DNA concentration test in sambar deer had an average of 12.375 ng/uL, with an average DNA purity test = 1.34, with 10 samples divided into 3 haplotypes. The level of genetic diversity of sambar deer from all samples is $\pi = 0.01745 \pm 0.00380$, and haplotype diversity of Hd = 1,000 ± 0.045. Based on the phylogenetic tree, there are two parts: the Asian and Kalimantan regions. The conclusion of the current study showed that sequencing analysis of Sambar deer shows relatively high diversity and is a reasonable basis for performance selection and development of modern Sambar deer breeding.

Keyword : *Cervus unicolor* brookei; D-loop; mitochondrial DNA; Genetic diversity; Haplotipe

INTRODUCTION

Genetic diversity refers to the variation that occurs in the genome of a species caused by various factors. (Suparningtyas et al., 2018). Genetic diversity provides important information in conversion strategies, domesticated animals' population enhancement, and their adaptation to specific environments. (Liu et al., 2022). Genetic diversity is essential in improving genetics and facilitating adaptation to changing livestock breeding objectives. Diversity measurement is an important step in establishing genetic relationships. It is a crucial germplasm characterization and conservation process to more effectively control genetic erosion, design sampling strategies, and inform the breeding process.

Germplasm molecular data is helpful for individual identification, population diversity, and structure or for studying the evolution of related species (Agisimanto & Supriyanto, 2007). Sambar deer, as a native germplasm of East Kalimantan authorized by the Ministry of Agriculture Number 2838/Kpts/LB.430/8/2012, is an animal with a protected status that needs to know its genetic diversity. Sambar deer is a large deer species commonly inhabited in Asia. Sambar deer live in hot tropical and sub-tropical forests, with an altitude of 2,600 meters above sea level (above sea level) (Whitehead, 1993).

The genetic diversity of Sambar deer germplasm is still relatively high. However, basic data on the genetic diversity of Sambar deer has not been updated, and observations need to be made in the field of mitochondrial DNA. Mitochondrial DNA is a small part of the total DNA content in eukaryotic cells. (Taylor & Turnbull, 2005). Mitochondrial DNA is essential for biochemical assays on highly purified mitochondrial fractions to identify roles in evolutionary studies. This study was conducted with DNA observations to determine the genetic diversity of Sambar Deer and generate valuable insights into the relationships between populations, races, and species.

One way to determine the genetic diversity of Sambar deer is by providing molecular markers in the form of control regions (*displacement* (D) *loops*). The mitochondrial D-loop is a three-stranded region found in many mitochondrial genomes' central non-coding region (NCR) and forms a stable merger of short DNA strands. (Nicholls & Minczuk, 2014). Amplify regions can be done through the PCR technique stage. Designing primers in determining the size of amplicons and regions in complex genomes. (Rianti et al., 2021).

In this study, what was done from previous research was to compare the genetic diversity of Sambar Deer from South Sumatra and East Kalimantan. (Wirdateti, 2012), but previous research has not highlighted the repetitive side of the DNA chain. Therefore, this study aims to look at mitochondrial DNA sequencing of the D-Loop region control to obtain data on the level of genetic mutation and diversity of Sambar Deer (*Cervus unicolor*).

MATERIALS AND METHODS

Sampling

Sampling of sambar deer DNA using blood samples taken from (UPTD) Pembibitan Ternak dan Hijauan Pakan Ternak, Penajam Paser Utara, East Kalimantan with a total sample of 10 individual sambar deer consisting of 2 male sambar deer and eight female sambar deer. Blood sampling was done through the jugular vein using a vacuum cleaner tube inserted with EDTA with a volume of 5 ml.

DNA Extraction, Concentration, and Amplification

The DNA extraction process used the Genomic DNA Mini Kit (Blood/cultured cell) Geneaid. The extraction results in the form of DNA were then observed quantitatively using an IMPLEN NP80 touch Nanophotometer machine by adding 1 uL of Elution Buffer (EB) at the beginning of use and calculating the concentration of DNA at 260 and 280 nm and the purity of DNA with an absorbance ratio of A260/280. The primers used in this study were mtDNA amplified control region primers, namely Forward 5'-AAA CCA GAA AAG GAG AGC AAC-'3 and reverse 5'-TCA TCT AGG CAT TTT CAG TGC C-'3(Dauzery & Randi, 1997)(Wirdateti, 2012). PCR reactions on all samples used a volume of 30 µL, performed with an Eppendorf x50s PCR machine set to a temperature PCR program of 94℃ initial denaturation (2 minutes), 94℃ denaturation (1 minute 30 seconds, 35 cycles), 59.0℃ annealing (1 minute), 72℃ extension (1 minute), 72℃ final extension (5 minutes), and 4℃ colling down. The electrophoresis with 2% agarose gel media was run using a Mupid Exu Electrophoresis machine with a 100 Volt voltage power supply for 60 minutes. The electrophoresis results will be read on UV Gel Documentation (UVITEC Platinum V10) (Yuliati et al., 2019).

Sequence Data Analysis

The sequencing process was done with DNA alignment on comparison (BLAST analysis) with deer sequence data on the NCBI website http://blast.ncbi.nlm.nih.gov/ with GenBank number MF177016.1. Data analysis of the genetic diversity of sambar deer was carried out with the NCBI genome sequence method, which included observations of genetic diversity and polymorphism, mitochondrial DNA/Haplotype arrangement, and genetic mutations using Bioedit 7.7.1 and DNAsp 5.1 software. Genetic distance and mutations were analyzed using the Neighbor-joining (NJ) phylogenetic method with the Tajima-nei model and MEGA (*Molecular Evolutionary Genetics Analysis*) software, MEGA 11. The results of the data analysis obtained will then be analyzed descriptively.

RESULTS AND DISCUSSION

DNA Concentration and Purity Test

The results of the analysis showed that sambar deer blood DNA samples had an average purity test of 1.342 and an average concentration test of 12.375 ng/µL with the optimum DNA purity value at wavelengths A260/A280 in the range of 1.8-1.9, while RNA ranged from 1.9-2. So, the range of DNA extraction results is 1.8-2, as shown in Table 1. Analysis of isolated DNA to test the purity of DNA was carried out using a NanoDrop Spectrophotometer machine to measure the concentration and purity values with a wavelength of A260/A280.

Table 1. DNA Concentration and Purification Test Measurements

	Concentration (ng/µL)	Purification (A260/A280)
Average	12.375	1.342
Average metode 1	5.99*	36.21*
Average metode 2	6.33*	9.22*
Average metode 3	17.54*	2.84*

Desc : *source of (Pratama & Arisuryanti, 2022)

The composition of the five nucleotides that make up DNA or RNA with absorbance readings at A260/A280 wavelengths shows a value that varies, including guanine (1.15), adenine (4.50), cytosine (1.51), uracil (4.00), and thymine (1.47) (Utami et al., 2023). DNA isolation results obtained by DNA concentration test in the 6.900-24.350 ng/uL (average = 12.375 ng/µL). The results of DNA purity test data in the range of 0.9-1.5 (average = 1.34) and complete data are presented in Table 1. The pure DNA molecules' absorbance ratio is still far from the range of the DNA purity test, which ranges from 1.8-2.0, and it was concluded that the results of DNA extraction are not included in the optimum concentration. Previous research with several methods and the same kit showed the average concentration in method 1 with a concentration of 5.99 and purity of 36.21, method 2 with a concentration of 6.33 and purity of 9.22, and method 3 with a concentration of 17.54 and purity of 2.84 (Pratama & Arisuryanti, 2022). For the concentration test, the data obtained is 6.900-24.350, which is still relatively low from the absorbance ratio. However, the extraction results can be tested using a PCR tool so that the concentration and purity value of DNA depend on the sensitivity of a PCR tool. Some previous researchers have shown low DNA quality and purity with non-invasive blood sampling (Ando et al., 2020).

Temperature Gradient and PCR Results

The results of PCR amplification in 12 individuals consisting of 4 DNA templates from electrophoresis show that the PCR annealing temperature gradient is 59.0 ℃ found in healthy 9, in determining the temperature gradient results for the bulk of the brightest DNA band thickness seen in Figure 1.

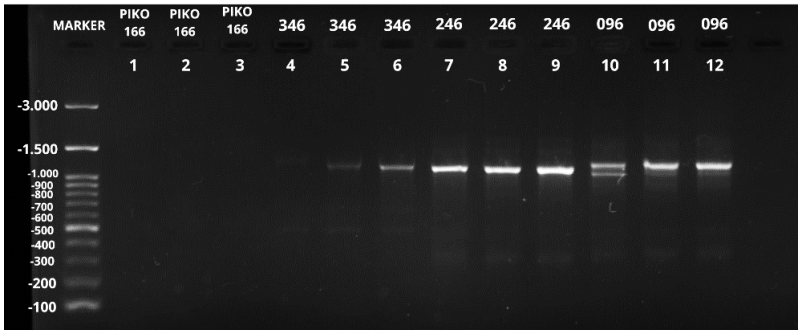

Figure 1. Annealing Well Gradient PCR Results of Deer CR-F and CR-R Primers

Primer parameters are melting temperature Tm, percentage of G and C (%GC), 3'dimer, stability, repeats, and hairpins. The forward primer has 21 bases with a 5'-AAA CCA GAA AAG GAG AGC AAC-'3 base sequence. The reverse primer has 22 bases with a base sequence of 5'-TCA TCT AGG CAT TTT CAG TGC C-'3 so that amplification of the D-loop region of mtDNA in sambar deer produces a target fragment with a sequence length of 1,200 bp (*base pairs*) with a thick visible DNA band (Wi et al., 2014). The PCR results showed that there were seven double helixes in the PCR results consisting of wells 1 (429), 2 (283), 4 (309), 6 (385), 8 (235), 10 (096), and 13 (379).

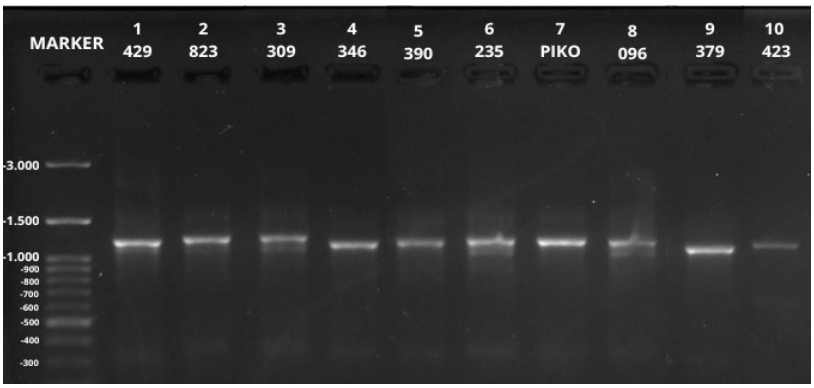

Figure 2. Mass PCR Results of FIX Anneling Deer CR-F and CR-R Primers and Indel Mutation Results

PCR results show that the DNA band's thickness looks thick with a high DNA concentration, so amplification is successful. Although not all DNA isolation samples provide results that match the primer product, the amplification primer is still considered successful. The best DNA band thickness is found in sample wells 1(429), 2(283), 3(035), 4(309), 5(346), 7(390), 8(235), 9(Piko), 10(096), 11(246), 12(389), 15(423), and 16(402), while the thickness of the DNA band that looks thin is in sample wells 6(385), 13(379), and 14(384). Genetic variation is the essential information in developing strategies for conservation breeding, management, and sustainable utilization of genetic resources (Aulia et al., 2021).

Genetic Diversity

The results of all samples amplified at the gene control region successfully produced amplicons of 1,200 base pairs. Although the concentration of amplicons varied, the length was consistent, as expected, as shown by the gel visualization. Furthermore, after data preparation, we found that the length of the obtained sequence was ±1,200 bp, but the result that was read and used for analysis was 466 bp. The sequence results will be analyzed using the program Bioedit ver. 7.7.1 and DNAsp 5.1 by conducting further analysis through the sequence identity checker against the NCBI database.

Table 2. Sambar Deer Haplotypes Based on Repetitive Sequences in the D-loop Region

Haplotypes	Sequence Motive	N	Frequency
A	$(GGACATATTATGTATAATAGTACATAAAATTAATGTATTA)_1$	2	0.2
B	$(GGACATATTATGTATAATAGTACATAAAATTAATGTATTA)_2$	6	0.6
C	$(GGACATATTATGTATAATAGTACATAAAATTAATGTATTA)_3$	2	0.2
	Total	10	1.0

Desc : N: Number of Individuals

Analysis showed that all samples could be grouped into three haplotypes with three sequence motifs. The base length between 10 samples and 1 GenBank formed a corresponding sequence length of 466 bp. There are repetitive sequences in the sequence base sequence from 15,190 bp to 15,309 bp. There are three types of repetitive sequence motifs in Table 2. Repeats in the sequence were observed to be duplicated with varying numbers of copies, ranging from one to three times in the sambar deer population (Ghazi et al., 2021).

Table 3. Sambar Deer in Each Haplotype

Haplotype	N_{Hap}	Variants (Eartaq)
A	2	A1 (429) & A3 (346)
B	6	B1 (309), B2 (390), B3 (235), B4 (Piko), B5 (096) & B6 (379)
C	2	C1 (823) & C2 (423)

Desc : H: haplotype; Nhap: Number of Haplotype

Phylogeographic tree reconstruction was carried out by comparing all sambar deer (*Cervus unicolor brookei)* sequence samples from this study with sambar deer (*Cervus unicolor brookei*) sequences obtained from GenBank (NCBI) with access number MF177016.1. Moreover, compared with outgroups obtained from GenBank (NCBI), namely other types of Sambar deer (*Cervus unicolor*) with access numbers MF177000.1, MF176992.1, KY117576.1, KX156946.1, and OR077747.1 and Elaphus deer (*Cervus elaphus*) with access numbers MF872248.1, MF872249.1, and AB245427.2. The selection of GenBank at the access number is to find a match in GenBank with all sequence samples with the selection through the same primer. Genetic markers and sample size in the study, showed there were 10 variations with 10 sambar deer blood samples. Further analysis showed that the three haplotypes had a high haplotype (Hd) and diversity level. Three haplotypes found a different pattern from Hd and π, where the genes in sambar deer follow the pattern of maternal lineage heredity and do not experience recombination.

Table 4. Genetic Diversity of Sambar Deer Based on (466 BP) Sequences

Haplotype	N_V	N_{ind}	Hd	Π	Fu'st statistik	Tajima D test
A	2	2	1,000±0,500	0,01087±0,00486	-	-
B	6	6	1,000±0,096	0,01780±0,00407	-1,285 ^{ns}	-0,14072 ^{ns}
C	2	2	1,000±0,500	0,00870±0,00435	-	-
Total	10	15	1,000±0,045	0,01745±0,00380	-4,188 ^{ns}	-0,47492 ^{ns}

Desc: N_V: Number of Variations (types) of Sequences, Nind: Number of Individuals, Hd: Haplotype Diversity, π: Nucleotide Diversity

Therefore, the sambar deer sample genes were subjected to phylogenetic analysis. The highest nucleotide diversity (π) is π = 0.01780 ± 0.00417 in haplotype B, while the highest haplotype diversity (Hd) is Hd = 1.000 ± 0.500 in haplotype C and haplotype A. The most haplotypes are found in haplotype B, with six haplotypes analyzed.

The level of genetic diversity of sambar deer from all samples is π = 0.01745±0.00380 and haplotype diversity is Hd = 1.000±0.045, so the results obtained are low compared to the results of Wirdateti with haplotype diversity, Hd = 0.9870 and π = 2.931 ± 0.260 and relatively high compared to other Cervidae species, such as Chital (*Axis axis*) with haplotype diversity (Hd) 0.461±0.07 and nucleotide diversity (π) 0.0023 ±0.00035 (Hill et al., 2019); Chital (*Axis axis*) with haplotype diversity (Hd) 0.400 (Pratama et al., 2023); Deer Timorensis (*Cervus* timorensis) with haplotype diversity (Hd) 0.056 and nucleotide diversity (π) 0.039 (Iman et al., 2024); Chinese water deer (*Hydropotes inermis inermis*) with haplotype diversity (Hd) 0.923 ± 0.025 and nucleotide diversity (π) 1.318 ± 0.146% (Hu et al., 2006); and Muntiacus muncak with a haplotype diversity (h) of 0.862 and a nucleotide diversity (π) of 0.0056 (Wu & Fang, 2005).

Information obtained in knowing genetic variation is essential to predict the state of the sambar deer population (*Cervus unicolor brookei*) in the breeding area. DNA markers provide information in observing population structure and developing conservation management and accurate indicators in seeing population structure (Avise et al., 1987)(Hisheh et al., 1998)(Zein, 2009). Haplotype diversity categories ranged from 0.8-1 in the high category, 0.5-0.7 in the medium category, and 0.1-0.4 in the low category. (Nei, 1987). Categories of haplotype diversity values include 0-0.5, a low category, and 0.5-1, a high category. (Hobbs et al., 2013).

Genetic Mutations

In the 449 base sequence region, there are 21 polymorphic nucleotide sites and nine types of sequences (haplotypes), namely haplotypes A1, A2, B1, B2, B3, B4, B5, B6, C1, and C2 in Table 5 and compared with GenBank sequences MF177016.1, MF176992.1, and MF177000.1. The polymorphic nucleotide base sequence is located at sites 3-442 and comprises substitutions and insertions.

Results showed 56 mutations in all sambar deer haplotypes, as shown in Table 5. Based on the sequence of nucleotide bases, it was found that nucleotide substitutions were used to characterize nine haplotypes when compared with *Cervus unicolor* (MF177016.1), *Cervus unicolor* (MF176992.1), and *Cervus unicolor* (MF177000. 1) there are polymorphic sites consisting of singleton variation of 9 sites (100, 104, 172, 265, 341, 358, 367, 377, 442), parsimony variations of 10 sites (3, 50, 63, 98, 131, 177, 314, 316, 406, 408), and parsimony variation of two variations of 1 site (317). The nucleotide insertion mutation was found in nucleotide insertion A in the 375th base sequence in the C1 and C2 haplotypes. Mutations occur along the sequence base position with different mutation patterns.

Table 5. Sequence Variation in 9 mtDNA Control Region haplotypes and 2 GenBank in Sambar Deer with GenBank reference sequence MF177016.1 bp base sequences.

No.	Haplotype	Nucleotide Sequence Number										
				11	11	12	33	33	33	33	44	4
		5	69	00	37	76	11	14	56	77	00	4
		30	38	04	12	75	46	71	87	57	68	2
1.	*Cervus unicolor* A1	AC	A.	T.	..	..	..	..	..	-.	GC	C
2.	*Cervus unicolor* A3	AC	AG	..	..	..	C.	..	..	-.	GC	.
3.	*Cervus unicolor* B1	A.	..	..	A.	C.	..	C.	..	-.	..	.
4.	*Cervus unicolor* B2	AC	..	..	.A	.G	.A	A.	A.	-A	GC	.
5.	*Cervus unicolor* B3	..	..	..	A.	..	C.	..	..	-.	..	.
6.	*Cervus unicolor* B4	A.	..	..	..	C.	..	..	..	-.	..	.
7.	*Cervus unicolor* B5	..	..	..	A.	..	..	C.	..	-.	..	.
8.	*Cervus unicolor* B6	AC	..	.A	..	..	.A	.T	..	-.	GC	.
9.	*Cervus unicolor* C1	AC	AG	..	..	..	C.	..	..	A.	GC	.
10.	*Cervus unicolor* C2	AC	AG	..	..	..	C.	..	..	A.	GC	.
11.	*Cervus unicolor* MF176992.1	..	..	..	..	..	..	..	..	-.	..	.
12.	*Cervus unicolor* MF177000.1	A.	..	..	..	C.	..	..	.A	-.	..	.

Phylogenetic Tree

Neighbor-Joining (NJ) described the phylogeny tree of the Tajima Nei model of 10 haplotypes of sambar deer. The phylogenetic tree was analyzed by dividing it into three haplotypes. Phylogeographic tree reconstruction was carried out by comparing 10 samples of sambar deer (*Cervus unicolor*) haplotype sequences from this study with sambar deer (*Cervus unicolor*) sequences obtained from GenBank (NCBI) with access numbers MF177016.1, MF176992.1, and MF177000.1. In field sample collection, the deer sampled were recorded by looking at appearance and quantitative measurements to determine which deer belonged to which grouped region. Sambar deer included in the Bornean Lineage region group are morphologically very different from the Asian Lineage region sambar deer have morphology on.

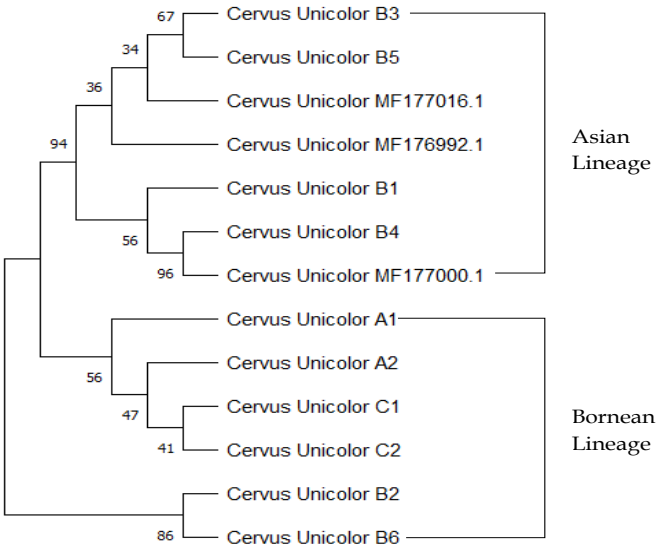

Figure 3. Phylogenetic Tree of 10 Sambar Deer Haplotypes and 3 GenBanks Based on Alligment Region

In each regional group, the Asian part has haplotypes consisting of B1, B3, B4, and B5; there is still one with sambar deer quoted from Genbank (NCBI), namely MF176992.1, MF170000.1, and MF177016.1. Meanwhile, in the Kalimantan region, which consists of A1, A2, B2, B6, C1, and C2, these Eartag variants can represent several other Eartag Variants with the same haplotype. Sambar deer are currently subdivided into five subspecies: R. u. unicolor (India, Nepal, Bangladesh, and Sri Lanka), R. u. brookie (Borneo), R. u. cambojensis (mainland Southeast Asia, from South China/Hainan and Myanmar to Peninsular Malaysia), R. u. equine (Sumatra and Mentawai), and R. u. swinhoei. (Martins et al., 2018).

Genetic Distance

Genetic distances were generated by clustering on each haplotype obtained, and comparative sambar deer sequences obtained in GenBank were analyzed on neighbor-joining distances with bootstrap calculations 1000 times using MEGA 11 software. Standard genetic distances can develop genetic measures with evolutionary time when the effects of mutations and genetic drift are considered (Nei, 1972). The phylogenetic tree illustrates the overall genetic distance of sambar deer samples. Based on the study's results, the value of genetic distance in haplotypes is 0.000 to 0.031. The smallest or low genetic distance is haplotype C2-C1 and MF176992.1 (GenBank)-MF177016.1 (GenBank), while the highest genetic distance is haplotype B2-MF177000.1 (GenBank), haplotype B2-B1, haplotype B3-B2, and haplotype B5-B2 Figure 4. The lower the genetic distance, the lower the genetic dissimilarity between individuals or the closer the kinship between individuals (Rahayu, 2010).

Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13
MF177016.1													
MF177000.1	0,007												
MF176992.1	0,000	0,007											
Haplotipe A1	0,020	0,022	0,016										
Haplotipe A2	0,013	0,015	0,014	0,011									
Haplotipe B1	0,009	0,007	0,009	0,024	0,018								
Haplotipe B2	0,027	0,031	0,023	0,027	0,024	0,031							
Haplotipe B3	0,002	0,011	0,002	0,025	0,018	0,009	0,029						
Haplotipe B4	0,004	0,002	0,005	0,020	0,013	0,004	0,029	0,009					
Haplotipe B5	0,004	0,013	0,005	0,027	0,020	0,007	0,029	0,002	0,011				
Haplotipe B6	0,020	0,024	0,018	0,020	0,018	0,027	0,020	0,022	0,022	0,024			
Haplotipe C1	0,020	0,025	0,016	0,013	0,009	0,027	0,022	0,022	0,022	0,024	0,018		
Haplotipe C2	0,018	0,020	0,016	0,016	0,004	0,027	0,029	0,022	0,018	0,025	0,022	0,009	

Figure 4. Genetic Distance across Haplotypes and GenBank Sequences

The neighbor-joining method is essential in the principle of taxonomic grouping based on the evolutionary distance value contained in the phylogenetic tree with *operational taxonomy units*. So if the smaller the number produced by the distance, the genetic distance with other haplotypes is closer, while the greater the number produced by the distance, the distance with other haplotypes is further away. Genetic distance is considered low if it has a value between 0.010 and 0.099, medium 0.01 to 0.99, and high 1.00 to 2.00 (Nei, 1972).

CONCLUSION

The conclusion of this study indicates that the genetic diversity of the sambar deer (Cervus unicolor) population in East Kalimantan, analyzed through mitochondrial DNA D-loop sequencing, is relatively high. Additionally, the demographic structure revealed by the D-loop sequencing differs from populations in other regions.

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