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Research Article

Preliminary Study on Phylogenetic Analysis of Sakub Sheep from Brebes Regency Based on the Mitochondrial DNA (mtDNA) Displacement Loop (D-Loop) Gene Sequence

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Abstract | The mitochondrial DNA (mtDNA) Displacement Loop (D-Loop) gene is commonly used in genetic studies due to its variability and ability to provide insights into phylogenetic relationships. Sakub sheep, a local Indonesian breed from Brebes Regency in Central Java, have not yet been studied using D-Loop gene sequences to determine phylogenetic relationships. This research aims to analyze the genetic relationships of Sakub sheep with other breeds and identify their haplogroup. DNA isolates from Sakub sheep were amplified using specific primers (Alek-DLF 5'-GAAGAAGCTATAGCCCCACT-3' and Alek-DLR 5'-GATTCGAAGGGCGTTACT-3'), sequenced, and analyzed with MEGA software. Comparative data was sourced from NCBI GenBank. Sequence alignment revealed 1180 base pairs, with 1131 monomorphic sites, 50 polymorphic sites, 11 segregating sites, and 39 parsimony-informative sites. Genetic distances among Sakub samples ranged from 0.000 to 0.039, indicating a close phylogenetic relationship among individuals. Two samples (SKB.4 and SKB.8) were classified under haplogroup A, while six (SKB.1, SKB.2, SKB.3, SKB.5, SKB.6, SKB.7) were under haplogroup B. The findings suggest that Sakub sheep predominantly belong to haplogroup B and share close phylogenetic relationships with other local Indonesian breeds as well as international breeds such as Merino, Suffolk, and Texel.

Keywords | D-Loop gene, Phylogenetic, Polymerase chain reaction (PCR), Sakub sheep

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INTRODUCTION

As an archipelago, Indonesia boast a rich diversity of natural resources, including various local sheep breeds. Local sheep are those bred and raised by communities in specific regions across Indonesia, where they have adapted well to their respective environments (Ibrahim *et al*., 2020). These local breeds serve as a valuable genetic resource that must be preserved, including the Sakub sheep, which thrive on the slopes of Mount Slamet in Brebes Regency, Central Java, particularly in Pandansari Village (Paguyangan District) and Wanareja Village (Sirampog District) (Nurasih *et al*., 2023;). Sakub sheep result from crossbreeding Texel, Suffolk, Merino, and other local sheep, producing a superior, unique, and uniform breed. This breed was officially recognized as a distinct genetic lineage by the Indonesian Ministry of Agriculture of Indonesia under Decree No. 882/KPTS/PK.010/M/12/2022 (Ministry of Agriculture, 2022).

Sakub sheep are characterized by brown, white, or brown-white coats, relatively thick tails, convex-shaped heads, and a mix of horned and hornless individuals (Nurasih *et al*., 2023). They exhibit strong potential for meat production, as indicated by their morphometric traits, including chest circumference, body length, and height (Meliana *et al*., 2024).

Mitochondrial DNA (mtDNA) is an informative genetic marker for phylogenetic studies due to its small size, high copy number, maternal inheritance, and higher mutation rate compared to nuclear DNA (Koshkina *et al*., 2023; Wirdateti and Semiadi, 2017). Genetic diversity and relationships can be analyzed by sequencing the mtDNA D-Loop and Cytochrome B (Cyt B) genes (Koshkina *et al*., 2023). The hypervariable D-Loop region, a control region involved in replication and transcription, mutates four to five times faster than other mtDNA areas, making it ideal for phylogenetic studies (Wirdateti and Semiadi, 2017). The D-Loop comprises two regions, HVR1 (positions 16,000–16,569) and HVR2 (positions 1–400) (Hartatik, 2014).

Genetic profiles based on the D-Loop gene have been studied for several Indonesian sheep breeds, including Thin-tailed, Fat-tailed, Batur, Wonosobo, Garut, and Priangan sheep (Ibrahim *et al*., 2020). However, no phylogenetic analysis has yet been conducted on Sakub sheep using the D-Loop gene. This study aims to analyze genetic relationships and determine the haplogroup of Sakub sheep based on mtDNA D-Loop sequences.

MATERIALS AND METHODS

Ethical Approval

The use of Sakub sheep in this study was conducted in accordance with research methods reviewed and approved by the Ethical Clearance Commission of the Veterinary Medicine Faculty, Universitas Gadjah Mada (UGM), and validated through the Ethical Clearance Statement No: 086/EC-FKH/Int./2022 on October 15th, 2022.

Sample Collection

Eight samples were collected from Sakub sheep farms located in Pandansari Village and Wanareja Village, Brebes Regency, Central Java Province. The samples comprised at least 10 strands of Sakub sheep hair per individual, collected from the neck and rump areas, ensuring the follicles were included. Each hair sample was cut to approximately 0.5–1.0 cm long and stored in microtubes (Safitri, 2023).

DNA Isolation

DNA isolation was performed following the Geneaid gSYNC™ DNA Extraction Kit Quick Protocol (Geneaid Biotech, Taiwan). The DNA isolation process included cell lysis, DNA binding, washing, and elution.

Polymerase Chain Reaction (PCR)

The DNA isolates were amplified using the forward primer (Alek-DLF: 5'-GAAGAAGCTATAGCCCCACT-3') and reverse primer (Alek-DLR: 5'-GATTCGAAGGGCGTTACT-3'), resulting in a PCR product of 1397 bp (Ibrahim *et al*., 2020). The PCR amplification was performed in a 50 µl reaction mixture containing 25 µl of 2x MyTaq HS Red Mix (Meridian Bioscience, US), 19 µl of ddH_2O, 3 µl of DNA isolate sample, and 1.5 µl each of forward and reverse primers, using a thermal cycler (Cleaver GTC96S, Cleaver Scientific Ltd, England).

The protocol began with a pre-denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation, annealing, and elongation. Denaturation was set at 94°C for 30 seconds, annealing at 53°C for 40 seconds, and extention at 72°C for 90 seconds, followed by a final extention step at 72°C for 8 minutes.

Agarose Gel Electrophoresis and Sequencing

The PCR products were visualized using 1.5% agarose gel electrophoresis. The running step was carried out at 100V for 30 minutes, and the results were observed using a UV transilluminator. Subsequently, PCR products from eight samples were sequenced using the Sanger sequencing method, following the procedures of PT Genetika Science Indonesia and the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada.

Data Analysis

The sequencing results were analyzed using the Molecular Evolutionary Genetic Analysis (MEGA) software version 11.0.13. The sequence alignment was performed using the Clustal W method. The nucleotide genetic distances were analyzed using the Pairwise distance method and the Ki-

mura 2-parameter model. The phylogenetic tree was constructed using the Neighbor-Joining method with a bootstrap test of 1000 replicates and the Kimura 2-parameter model. Phylogenetic tree analysis used reference samples from domestic sheep (*Ovis aries*) obtained from NCBI GenBank and local Indonesian sheep (Ibrahim, 2021). The reference samples for phylogenetic analysis are presented in Table 1.

Table 1: Reference of comparison samples in phylogenetic analysis.

Group	Reference
Ovis aries	**NCBI Genbank**
Haplogroup A	Accesion number HM236174 and HM236175
Haplogroup B	Accesion number HM236176 and HM236177
Haplogroup C	Accesion number HM236178 and HM236179
Haplogroup D	Accesion number HM236180 and HM236181
Haplogroup E	Accesion number HM236182 and HM236183
Local sheep	
Jawa Ekor Gemuk sheep	Code: JEG (Ibrahim, 2021)
Jawa Ekor Tipis sheep	Code: JET (Ibrahim, 2021)
Wonosobo sheep	Code: WSB (Ibrahim, 2021)
Priangan sheep	Code: PRG (Ibrahim, 2021)
Garut sheep	Code: GRT (Ibrahim, 2021)
Batur sheep	Code: BTR (Ibrahim, 2021)
Sapudi sheep	Code: SPD (Ibrahim, 2021)
Ovis aries (Breed Texel)	Accession number NCBI *Genbank* AY849210
Ovis aries Breed Suffolk	Accesion number NCBI *Genbank* MK174645
Ovis ammon	Accesion number NCBI *Genbank* HM236188
Ovis musimon	Accesion number NCBI *Genbank* HM236184
Ovis vignei	Accesion number NCBI *Genbank* HM236187
Ovis orientalis	Accesion number NCBI *Genbank* KF312238
Ovis canadensis	Accesion number NCBI *Genbank* MH094035
Ovis nivicola	Accesion number NCBI *Genbank* MH779626
Ovis dalli	Accesion number NCBI *Genbank* MH779627

RESULTS AND DISCUSSION

Amplification of the D-Loop Gene Using the PCR Method

The amplification produced a product (amplicon) of 1397 bp. The PCR products were visualized using 1.5% agarose gel electrophoresis, observed under a UV transilluminator, as shown in Figure 1. Wells 1-8 exhibited clear and relatively thick DNA bands. The clear and thick DNA bands indicate optimal DNA concentration (Iqbal *et al*., 2016). Based on the visualization results, the samples selected for sequencing included eight samples with the codes SKB.1, SKB.2, SKB.3, SKB.4, SKB.5, SKB.6, SKB.7, and SKB.8.

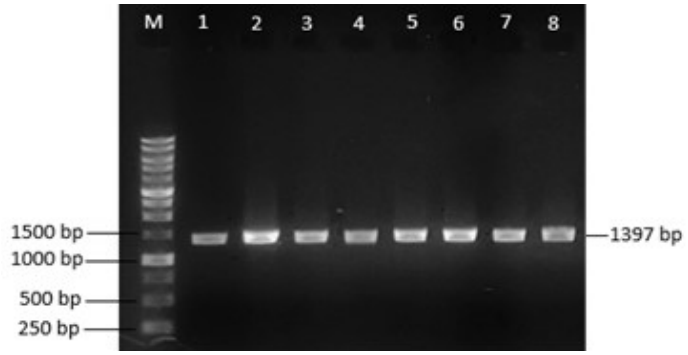

Figure 1: Visualization of D-Loop gene amplification results from Sakub sheep samples using 1.5% agarose gel. 1) SKB.1, 2) SKB.2, 3) SKB.3, 4) SKB.4, 5) SKB.5, 6) SKB.6, 7) SKB.7, 8) SKB.8, and M) DNA marker 1kb.

Nucleotide Sequence Analysis

The alignment results revealed a product of 1180 bp, based on the complete D-Loop gene sequence of *Ovis aries* obtained from NCBI GenBank with accession number HM236176.1. The average nucleotide composition percentages of thymine (T), cytosine (C), adenine (A), and guanine (G) in the D-Loop gene sequence of Sakub sheep are 29.5%, 23.0%, 33.1%, and 14.4%, respectively. The alignment results indicate 1131 monomorphic sites, 50 polymorphic or variable sites, 11 segregation or singleton sites, and 39 parsimony informative sites. The positions of the polymorphic sites are shown in Figure 2. The polymorphic sites indicate the presence of substitution mutations in the form of transition mutations, where there is a substitution between purine bases (A and G) or between pyrimidine bases (C and T). Substitution mutations are characterized by a nucleotide base change to another form within the DNA sequence, which can lead to evolution, consisting of transition and transversion mutations. Transition mutations occur due to the exchange between purine bases (A and G) or pyrimidine bases (C and T). In contrast, transversion mutations are caused by exchanges between purine and pyrimidine bases (Kartika *et al*., 2017).

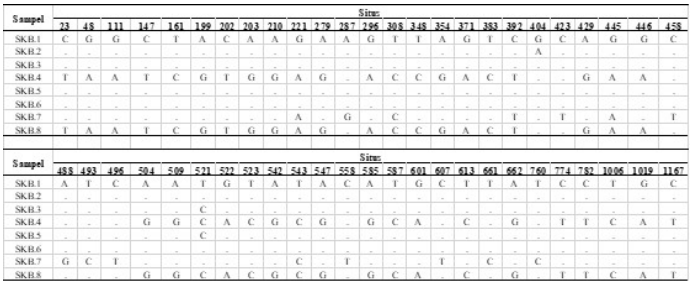

Figure 2: Position of polymorphic sites in Sakub sheep samples based on D-Loop gene sequence.

Genetic Distance

The genetic distance among Sakub sheep samples ranged from 0.000 to 0.039. The lowest genetic distance, indicated by a value of 0.000, is observed between the relationships of samples with the codes SKB.6 and SKB.1, SKB.5 and SKB.3, and SKB.8 and SKB.4. The most significant genetic distance, 0.039, is observed between samples SKB.4 and SKB.7, as well as between SKB.7 and SKB.8. The genetic distance between Sakub sheep samples is presented in Table 2. A genetic distance close to or equal to zero (0) indicates a close genetic relationship, while a genetic distance of one or more suggests a distant genetic relationship (Lestari *et al*., 2018; Ibrahim, 2021). Low genetic diversity indicates the presence of inbreeding, which can occur due to random or uncontrolled mating. The potential for inbreeding will continue if random mating occurs between animals with very close genetic distances, which can lead to a decline in biological fitness, such as increased homozygosity, mutation defects, expression of recessive alleles, and imbalances in gene flow (Lestari *et al*., 2018).

Table 2: Genetic distance between Sakub sheep samples.

Sample	SKB.1	SKB.2	SKB.3	SKB.4	SKB.5	SKB.6	SKB.7	SKB.8
SKB.1								
SKB.2	0.001							
SKB.3	0.001	0.002						
SKB.4	0.034	0.035	0.033					
SKB.5	0.001	0.002	0.000	0.033				
SKB.6	0.000	0.001	0.001	0.034	0.001			
SKB.7	0.013	0.014	0.014	0.039	0.014	0.013		
SKB.8	0.034	0.035	0.033	0.000	0.033	0.034	0.039	

Phylogenetic

The phylogenetic analysis utilized comparison samples from NCBI GenBank and references from local Indonesian sheep breeds (Ibrahim, 2021). The local sheep breeds included Jawa Ekor Gemuk (JEG), Jawa Ekor Tipis (JET), Wonosobo (WSB), Priangan (PRG), Garut (GRT), Batur (BTR), and Sapudi (SPD), with four samples each obtained from previous research (Ibrahim, 2021). Additionally, domestic sheep (*Ovis aries*) with haplogroups A, B, C, D, and E, as well as Texel and Suffolk sheep and wild sheep, were also included from NCBI GenBank.

The phylogenetic tree is divided into two main branches, separating domestic sheep (*Ovis aries*) from wild sheep. Based on the tree, the eight Sakub sheep samples are split into two haplogroups: two samples belong to haplogroup A (Asia-type sheep; sample codes: SKB.4 and SKB.8), and six samples belong to haplogroup B (Europe-type sheep; sample codes: SKB.1, SKB.2, SKB.3, SKB.5, SKB.6, and SKB.7). The Sakub sheep in haplogroup A are clustered in the same branch as Garut (GRT2, GRT3, GRT4), Priangan (PRG2, PRG3), Merino (HM236174), and Romney (HM236275) sheep. The Sakub sheep in haplogroup B are clustered in the same branch as Jawa Ekor Gemuk (JEG1, JEG2, JEG3, JEG4), Jawa Ekor Tipis (JET1, JET2, JET3, JET4), Wonosobo (WSB1, WSB2, WSB3, WSB4), Batur (BTR1, BTR2, BTR3, BTR4), Sapudi (SPD1, SPD2, SPD3, SPD4), Priangan (PRG1, PRG4), Garut (GRT1), Suffolk (MK174645), Texel (AY829410), Karakas (HM236176, HM236177), and *Ovis musimon* (HM236184). Based on this, the Sakub sheep samples fall under haplogroup B, representing European-type sheep. Sakub sheep show a close genetic relationship with local Indonesian sheep, Merino, Suffolk, and Texel breeds based on their proximity in the phylogenetic tree within the same branch. The phylogenetic tree is shown in Figure 3.

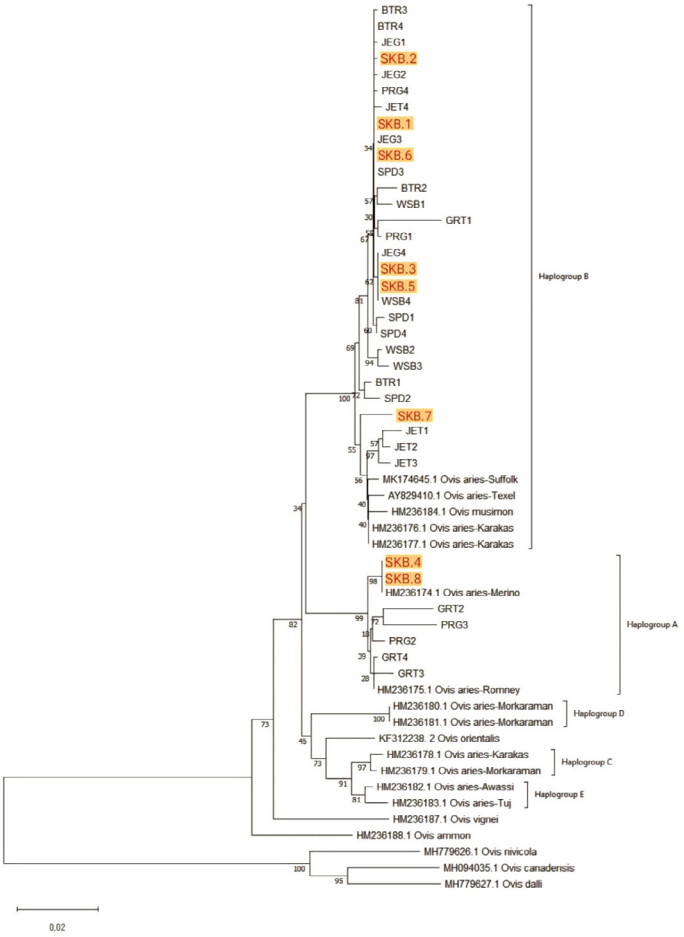

Figure 3: Phylogenetic tree based on D-Loop gene sequence of Sakub sheep samples and reference comparators.

Phylogenetics aims to represent organisms' evolutionary history by comparing species' genetic relationships through DNA or protein sequences. Phylogenetic classification is used to organize biodiversity information and has been universally applied in biological classification. Phylogenetics can also demonstrate the distribution of traits among lineages, whether morphological, biological, or molecular (Malabarba and Malabarba, 2019). In the mitochondrial

genome of the Ovis genus, domestic sheep (*Ovis aries*) are classified into five main haplogroups: A, B, C, D, and E (Koshkina *et al*., 2023; Meadows *et al*., 2005; Olivieri *et al*., 2012). Haplogroups A and B are the most widespread across various geographic regions. Haplogroup A represents Asian sheep breeds, while haplogroup B represents European sheep breeds found in high frequency (Olivieri *et al*., 2012). Haplogroup A is found in two breeds from Central Asia (Karakul/Kazakhstan and Gizarr/Tajikistan) and New Zealand (Romney, Coopworth, and Merino sheep). In contrast, haplogroup B is found in various European breeds, including the European mouflon (*Ovis musimon*). However, some European breeds belong to haplogroup A, and some Asian breeds, like Jawa Ekor Tipis, belong to haplogroup B. This suggests a high level of sheep transportation across geographic regions (Meadows *et al*., 2005).

CONCLUSIONS AND RECOMMENDATIONS

The genetic distance among Sakub sheep samples indicates a close genetic relationship, ranging from 0.000 to 0.039. Sakub sheep tend to be more closely related to European-type sheep, specifically haplogroup B. Additionally, Sakub sheep share a genetic lineage similar to other local Indonesian sheep breeds, as well as to Merino, Texel, and Suffolk sheep.

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NOVELTY STATEMENTS

This is the first study to analyze the phylogenetic relationship of Sakub sheep, a unique local breed from Brebes Regency, Indonesia, using mitochondrial DNA D-Loop sequences. The findings reveal that Sakub sheep predominantly belong to haplogroup B (European lineage) with some individuals in haplogroup A (Asian lineage), highlighting their mixed ancestry and genetic closeness to both local Indonesian and international sheep breeds such as Merino, Suffolk, and Texel.

AUTHOR'S CONTRIBUTIONS

Rana Ayuningtyas Adhi Puspita contributed to the conceptualization, investigation, sample collection, data analysis and interpretation, and original draft preparation. Alek Ibrahim was involved in conceptualization, investigation, sample collection, reviewing, and editing. Endang Tri Margawati contributed to reviewing and editing. Ismu Subroto was responsible for sample collection. Zaenab Nurul Jannah contributed to investigation, and research. Panjono was involved in reviewing and editing. Krisna Noli Andrian contributed to original draft preparation, reviewing, and editing. Medania Purwaningrum was involved in reviewing and editing. Aris Haryanto contributed to reviewing, editing, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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