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Genetic diversity and phylogenetic analysis of Indonesian local and commercial rabbit breeds based on mitochondrial D-loop

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ABSTRACT

This study aimed to evaluate the genetic diversity and phylogenetic relationships among four rabbit breeds raised in Central Java, namely Indonesian Local rabbit (LC), Hycole (HC), Hyla (HL), and New Zealand White (NZ), using mitochondrial D-loop region sequences. A total of 40 blood samples were collected from rabbits in three regencies in Central Java. DNA was extracted from each sample, followed by gene amplification, Sanger sequencing, and sequence analysis. A 906 bp fragment of the mitochondrial D-loop region was successfully amplified from all individuals. The overall genetic diversity was found to be low, with mean values of $k = 4.615$, nucleotide diversity (π) = 0.00509 ± 0.00092, and haplotype diversity (Hd) = 0.385 ± 0.070. Genetic distance analysis showed a close relationship among the breeds, ranging from 0.00349 to 0.00610. Two haplotypes (A and B) were identified, and both were shared across all breeds, indicating limited genetic differentiation. Phylogenetic tree reconstruction further demonstrated that the four breeds clustered closely together and were most likely derived from European lineages rather than distinct indigenous origins. In conclusion, rabbits raised in Central Java represent a genetically homogenous group with low diversity, highlighting the need for broader genetic resource management and conservation strategies to ensure long-term population sustainability.

Key words: Genetic distance, Haplotype variation, Maternal lineage, Nucleotide diversity, ,Rabbit breeding

INTRODUCTION

Rabbit farming in Central Java has been gaining popularity in recent years due to the rising demand for rabbit meat. In 2023, Central Java had the largest rabbit population for meat production in the country, accounting for 28.51% of the national total (Indonesian Ministry of Agriculture, 2024). The Indonesian Local rabbit is one of the most commonly reared breeds for meat production in Central Java. This breed is known for its adaptability to tropical climates and is categorized as a medium-sized breed. It is believed to have originated from European rabbit breeds introduced during the colonial period (Setiaji *et al*., 2023a). In addition, several exotic breeds have also been introduced to enhance meat production. The Indonesian Government, through the Indonesian Research Institute for Animal Production (IRIAP) imported two commercial broiler rabbit breeds, namely Hyla from China and Hycole from France (Brahmantiyo *et al.* 2017; Setiaji et al., 2023b). Moreover, the New Zealand White rabbit from the United States is also widely reared by farmers in Central Java. All of these imported breeds are well known for their superior production and reproduction traits (Setiaji *et al.,* 2022a).

However, obtaining purebred rabbits remains challenging. As most Indonesian rabbit farmers predominantly raise crossbred rabbits, resulting in the scarcity of purebred rabbits (Brahmantiyo *et al*., 2021; Setiaji *et al.,* 2022b; Setiaji *et al.,* 2024). This practice contributes to increased genetic diversity within rabbit populations. Therefore, studying the genetic diversity of commonly reared breeds in Central Java is crucial to support the development of appropriate breeding strategies. One current method for studying genetic diversity is through mitochondrial DNA (mtDNA) which is a genome located within mitochondria. This genome is inherited exclusively through the maternal line and does not undergo recombination (Ladoukakis and Zouros, 2017). The D-loop sequence, a non-coding region of mtDNA, is commonly used as a marker to assess genetic diversity. This region is highly variable due to higher substitution rate compared to other genes in mtDNA genome (Owuor, 2019). These characteristics make the D-loop suitable for evaluating genetic variation and reconstructing phylogenetic relationships based on maternal lineage.

Studies using D-loop sequence to assess genetic diversity on several rabbit breeds across different regions have been conducted in Kenya (Eastern, Western, Central Kenya) and Egypt (APRI, Black Baladi, Gabali, New Zealand, and Red Baladi breeds) (Owuor *et al*., 2019; Ahmed *et al*., 2022). However, studies on the genetic diversity of the Indonesian Local rabbit, Hycole, Hyla, and New Zealand White remain limited. Therefore, this study aims to analyze the genetic diversity, genetic distances, and phylogenetic relationships among these four rabbit breeds using mitochondrial D-loop sequence.

MATERIALS AND METHODS

Ethical Approval

The ethical approval of this study has been obtained by the Committee of Animal Research Ethics, Faculty of Animal and Agricultural Sciences Universitas Diponegoro (No. 59–01/A-01/KEP-FPP).

Sample Collection

A total of 40 rabbits comprising of 9 Indonesian Local rabbit (LC), 10 Hycole (HC), 11 Hyla (HL), and 10 New Zealand White (NZ) were collected from farms in Semarang, Temanggung, and Wonosobo Regency, Central Java, using purposive sampling. The selection criteria included bucks aged 3- 4 months with no genetic relationship to each other. Blood samples were collected via the central auricular artery using a 1 ml syringe and stored into EDTA tubes. DNA extraction was performed following the GeneJET Isolation Kit protocol (Thermofisher Scientific^{TM}).

Polymerase Chain Reaction and Sequencing

The D-loop sequence was amplified using primers designed by BLAST software based on the Indonesian Local rabbit sequence from Setiaji *et al.* (2023). The forward primer (F: 5’-AAACTCAAGTACTTCATCAG-3’) and reverse (R:5’-AAATACGCGGTGATTCT-3’) were used to amplify 1.204 bp fragment. The PCR reaction mixture contain 5 µl DNA template, 25 µl of MyTaq^{TM} HS Red Mix PCR Master Mix, 1 µl each of forward and reverse primers, and 18 µl of PCR water, all combined in a PCR tube. The amplification process was performed on a Bio-Rad T100 Thermal Cycler with the following conditions: initial denaturation at 95°C for 1 minute, denaturation at 95°C for 15 seconds, annealing at 48,5°C for 15 seconds, and extension at 72°C for 30 seconds, for a total of 35 cycles. The PCR results were stored in a freezer at -20°C. Visualization of PCR products was carried out by electrophoresis on 1% agarose gel at 100 volts for 30 minutes. Bands were observed under UV light using a GDS imaging system. Sequencing was performed by seqencing services (1st BASE, Singapore) using Sanger

Table 1. Reference Sequence for Haplotype Study

Haplotype	Accession Number	Breed
A	AF534100.1	Zika (Germany Great Line)
A	AJ293840.1	Domestic French Loop
A	KT030031.1	Black Baladi
B	AJ293839.1	Domestic French Loop
B	Z83366.1	Spanish Breed
B	U62925.1	Wild Rabbit (Australia)
D	U62927.1	Wild Rabbit (Australia)
K	KT029980.1	Black Baladi
K	KT030044.1	Red Baladi
K	KT030011.1	Gabali

Table 2. Reference Sequence for Phylogenetic Analysis

Accesison Number/Reference	Breed
PP357264.1	Jiuyi Mountain Rabbit
NC_001913.1	NZ1
sMN518689.1	Fujian Yellow Rabbit
X54172.1	Fauve de Borgougne
MH985853.1	NZ2
OZ175206.1	*Oryctolagus cuniculus*
MN953621.1	Chuanbai Rex
KR030070	*Lepus timidus*
Setiaji *et al.* (2023)	Indonesian Local rabbit

sequencing technique.

Data Analysis

The sequence polymorphism and genetic distances were analyzed using Molecular Evolutionary Genetics Analysis (MEGA)12 (Kumar *et al*., 2024). Sequence alignment was performed with the ClustalW program, followed by manual trimming. Genetic distances were calculated using the Kimura-2-parameter model. The number of segregating sites (S), singleton (Si) and parsimony (Pa) sites, nucleotide diversity ( ), haplotype diversity (h), and the number of haplotypes (nHap) were calculated using DnaSP v.6 software (Rozas et al., 2017). Tandem repeat sequence was calculated using Tandem Repeat Finder website (Benson, 1999).

Haplotype determination based on sequences from GeneBank as listed in Table 1. The phylogenetic tree was constructed using the Maximum Likelihood Method with 1000 bootstrap replication and applying Kimura-2-parameter substitution model in MEGA12 (Tamura *et al*., 2021). The analysis included 40 DNA sequences from this study along with 8 *Oryctolagus cuniculus* and 1 *Lepus timidus* sequences from GenBank for comparison (Table 2).

RESULTS AND DISCUSSION

The gene amplification results are shown in Figure 1, which demonstrates that the primers were able to amplify a target of approximately 1200 bp in length. After trimming, a 906 bp PCR product of the D-loop sequence was successfully obtained from the LC, HC, HL, and NZ samples. The results of the genetic diversity analysis are presented in Table 3. A total of 12 segregating sites (S) were identified from the 906 bp D-loop sequences analyzed in this study (Figure 2). HC

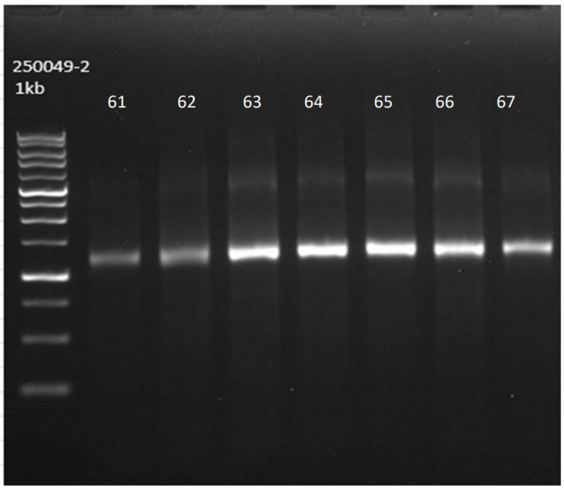


Figure 1. Visualization of D-loop gene amplification results

Table 3. Genetic Diversity Analysis in Four Rabbit Breeds

Breed	n	S	Si	Par	nHap	k	π±SD	Hd±SD
All	40	14	-	12	2	4,615	0,00509± 0,00092	0,385±0,070
LC	9	12	-	12	2	6,000	0,00662±0,0017	0,500±0,128
HC	10	12	12	-	2	2,400	0,00265±0,00204	0,200±0,154
HL	11	12	-	12	2	6,109	0,00674±0,00133	0,509±0,101
NZ	10	12	-	12	2	2,267	0,00471±0,00211	0,356±0,159

n = total number of individuals, S = segregating sites, Si = singleton, Pa = parsimony.

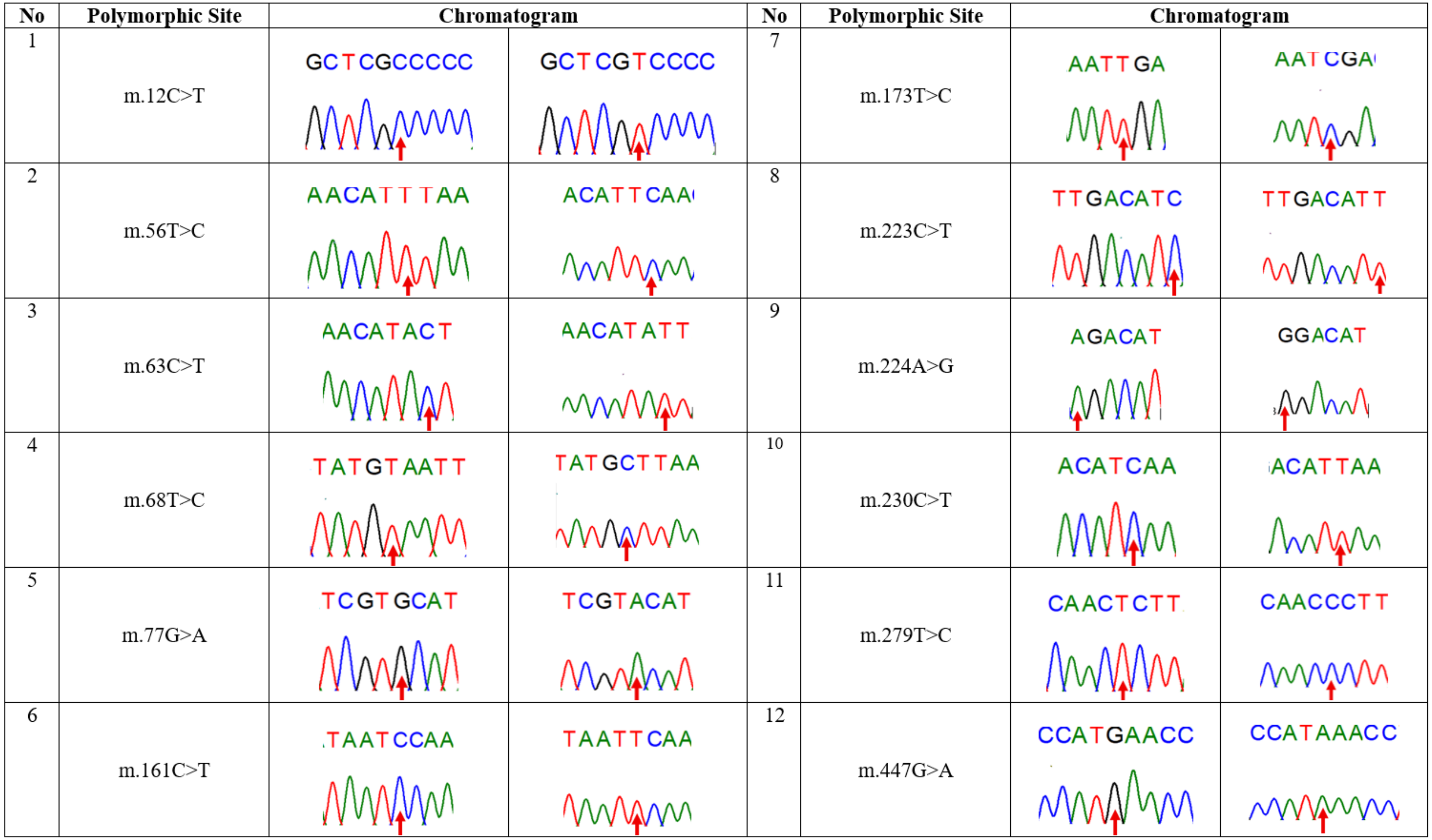
was the only breed showing singleton variable sites, indicating that only one out of ten HC rabbits samples had a different sequence variation. HC also showed the lowest average number of pairwaise differences (k), nucleotide diversity (n), and haplotype diversity (Hd) with values of 2,400; 0,00662±0,0017 and 0,200±0,154 respectively. In contrast, the highest values were observed in the HL breed which recorded k = 6,109, π = 0,00674±0,00133, and Hd = 0,509±0,101. Across all populations, the overall k= 4,615, π = 0,00509± 0,00092, and Hd= 0,385±0,070. Each breed generated two haplotypes. A 20 bp short tandem repeat (SR) sequence which located from position 706 to 906 was also identified. The repeat sequence, 5’-GCACGTACACCCGTACGCAC-3’ was repeated 10 times in all samples.

Genetic Diversity Analysis

The genetic diversity analysis in this study yielded lower results compared to the previous research. The number of segregating sites, haplotypes, average number of pairwise differences, nucleotide diversity, and haplotype diversity were all lower than the result from Ahmed *et al.* (2022) in Egyptian rabbit breeds and Owuor *et al.* (2019) in Kenyan rabbit populations. This indicates a low genetic diversity in the four rabbit breeds in Central Java.

This result could possibly happen due to the intense selection pressure for commercial rabbit such as Hyla, Hycole, and New Zealand White. Founder effect could happen due to intense selection (Long, 2003). Additionally, the Indonesian Local rabbit breed may have crossbred with commercial breeds or undergone inbreeding within its own population due to the lack of pedigree records, which could contribute to the reduce of genetic diversity (Owuor *et al.*, 2019; Kusumaningrum *et al.*, 2020).

The low genetic diversity observed in these rabbit breeds in Central Java has important implication. Limited genetic variation in the gene pool will reduce the probability to preserve gene who potentially hold desirable traits (Owuor,



No	Polymorphic Site	Chromatogram		No	Polymorphic Site	Chromatogram	
1	m.12C>T	GCTCGCCCCC	GCTCGTCCCC	7	m.173T>C	AATTGA	AATCGA
2	m.56T>C	AACATTTAA	ACATTCAA	8	m.223C>T	TTGACATC	TTGACATT
3	m.63C>T	AACATACT	AACATATT	9	m.224A>G	AGACAT	GGACAT
4	m.68T>C	TATGTAATT	TATGCTTAA	10	m.230C>T	ACATCAA	ACATTAA
5	m.77G>A	TCGTGCAT	TCGTACAT	11	m.279T>C	CAACTCTT	CAACCCTT
6	m.161C>T	TAATCCAA	TAATTCAA	12	m.447G>A	CCATGAACC	CCATAAACC

Figure 2. Chromatogram display showing mutations at 12 polymorphic sites

Table 4. Genetic Distance in Four Rabbit Breeds in Central Java

Breed	LC	HC	HL	NZ
LC				
HC	0,00492			
HL	0,00610	0,00525		
NZ	0,00537	0,00349	0,00561	

Table 5. Polymorphic Sites of D-loop Sequence in Four Rabbit Breeds

Breed	Samples	Polymorpic Sites											
		12	56	63	68	77	161	173	223	224	230	279	447
HC	HC1	C	T	C	T	G	C	T	C	A	C	T	G
	HC2	.	.	.	.	.	.	.	.	.	.	.	.
	HC3	.	.	.	.	.	.	.	.	.	.	.	.
	HC4	.	.	.	.	.	.	.	.	.	.	.	.
	HC5	.	.	.	.	.	.	.	.	.	.	.	.
	HC6	.	.	.	.	.	.	.	.	.	.	.	.
	HC7	T	C	T	C	A	T	C	T	G	T	C	A
	HC8	.	.	.	.	.	.	.	.	.	.	.	.
	HC9	.	.	.	.	.	.	.	.	.	.	.	.
	HC10	.	.	.	.	.	.	.	.	.	.	.	.
HL	HL1	.	.	.	.	.	.	.	.	.	.	.	.
	HL2	.		.	.	.	.	.	.	.	.	.	.
	HL3	T	C	T	C	A	T	C	T	G	T	C	A
	HL4	.	.	.	.	.	.	.	.	.	.	.	.
	HL5	.	.	.	.	.	.	.	.	.	.	.	.
	HL6	T	C	T	C	A	T	C	T	G	T	C	A
	HL7	T	C	T	C	A	T	C	T	G	T	C	A
	HL8	.	.	.	.	.	.	.	.				.
	HL9	.	.	.	.	.	.	.	.	.	.	.	.
	HL10	.	.	.	.	.	.	.	.	.	.	.	.
	HL11	T	C	T	C	A	T	C	T	G	T	C	A
NZ	NZ1	.	.	.	.	.	.	.	.	.	.	.	.
	NZ2	.	.	.	.	.	.	.	.	.	.	.	.
	NZ3	.	.	.	.	.	.	.	.	.	.	.	.
	NZ4	.	.	.	.	.	.	.	.	.	.	.	.
	NZ5	.	.	.	.	.	.	.	.	.	.	.	.
	NZ6	T	C	T	C	A	T	C	T	G	T	C	A
	NZ7	.	.	.	.	.	.	.	.	.	.	.	.
	NZ8	.	.	.	.	.	.	.	.	.	.	.	.
	NZ9	T	C	T	C	A	T	C	T	G	T	C	A
	NZ10	.	.	.	.	.	.	.	.	.	.	.	.
LC	LC1	T	C	T	C	A	C	C	T	G	T	C	A
	LC2	.	.	.	.	.	.	.	.	.	.	.	.
	LC3	.	.	.	.	.	.	.	.	.	.	.	.
	LC4	.	.	.	.	.	.	.	.	.	.	.	.
	LC5	.	.	.	.	.	.	.	.	.	.	.	.
	LC6	T	C	T	C	A	T	C	T	G	T	C	A
	LC7	T	C	T	C	A	T	C	T	G	T	C	A
	LC8	.	.	.	.	.	.	.	.	.	.	.	.
	LC9	.	.	.	.	.	.	.	.	.	.	.	.

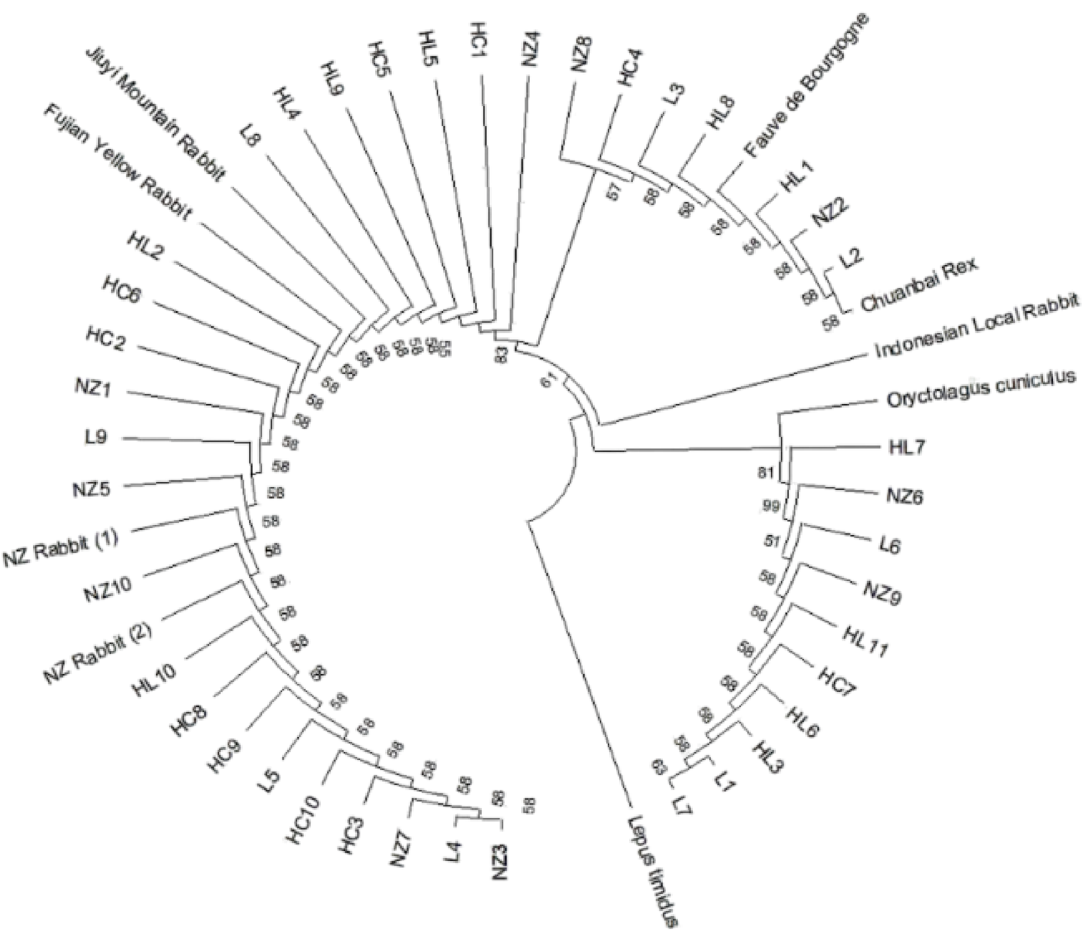


Figure 3. Phylogenetic Tree of Four Rabbit Breeds and other breeds from NCBI

Table 6. Haplotype Analysis of Four Rabbit Breeds

Haplotype	Samples	Total Samples
A	HC7, HL3, HL6, HL7, HL11, NZ6, NZ9, L1, L6,L7	10
B	HC1, HC2, HC3, HC4, HC5, HC6, HC8, HC9, HL1, HL2, HL4, HL5, HL8, HL9, HL10, NZ1, NZ2, NZ3, NZ4, NZ5, NZ7, NZ10, L2,L3, L4, L5, L8. L9	30

2019). Additionally, low diversity increases the risk of inbreeding, which can result into a decline in rabbit renal fat deposit and survival of the rabbit kits (Curik *et al.,* 2020; Piles *et al*., 2022). The Short Tandem Repeat (STR) sequence is consistent with Mignotte *et al*. (1990) who reported 20 bp sequence which repeated 10 times in Fauve de Bourgogne rabbits. In contrast, Setiaji *et al*. (2023) reported 11 repeats of the same sequence in Indonesian Local rabbits. This variation in repeat number could serve as a useful genetic marker for assessing diversity among rabbit breeds.

Genetic Distance, Haplotype Analysis, and Phylogenetic Relationship

Based on the genetic distance matrix presented in Table 4., the genetic distance among the four breeds ranged from 0,00349 to 0,00610. The lowest genetic distance was observed between NZ and HC at 0,00349, while the highest value of genetic distance was between HL and HC at 0,00610. Additionally, the genetic distance between haplotypes revealed that the divergence between haplotype A and haplotype B was 0,0134 in this study. Based on the polymorphic sites in Table 5 and haplotype analysis results shown in Table 6, two haplotypes were identified, haplotype A and haplotype B. Both haplotypes were present across of all the breeds. However, haplotype B was predominant with a total of 30 samples which comprising of 6 LC, 9 HC, 7 HL, and 8 NZ rabbits. The phylogenetic analysis illustrating the relationship between four rabbit breeds and GenBank sequences is presented in Figure 3. The analysis revealed two major clades which are *Lepus timidus* as the outgroup and *Oryctolagus cuniculus*. The *Oryctolagus cuniculus* clade further divided into three subclades, the haplotype A, haplotype B, and a single Indonesian Local rabbit from Setiaji *et al*. (2023). The majority of rabbit D-loop sequences from GeneBank clustered closely with haplotype B. However, the *Oryctolagus cuniculus* (accession number OZ175206.1) was more closely related to haplotype A.

The genetic distance analysis indicates that all of four breeds are closely related, as the genetic distance value is close to zero. Ibrahim *et al*. (2020) stated that genetic distance close or equal to zero suggest a close genetic relationship between populations. This finding is also supported by distance measurement based on morphometric characteristics, which showed that Hycole, Hyla, and New Zealand White have a close genetic relationship (Arafah *et al*., 2022).

Haplotype and phylogenetic analysis revealed a close phylogenetic relationship between haplotype A and B. Rabbits with the haplotype B rabbit in this clustered closely with Fauve de Bourgogne and the New Zealand White reference sequence from GenBank which represent the ancestor of commercial rabbits, including Hycole, Hyla, and New Zealand White (Queney *et al.,* 2002). Similarly, previous studies reported that rabbits in Kenya as well as the Gabali and Red Baladi breeds from Egypt are predominantly grouped within haplotype B. In contrast, native rabbits from Middle Egypt and New Zealand rabbit in Egypt, along with 10 of rabbits from various breeds in this study, were grouped in haplotype A, which originated from wild rabbit in the southwest of the Iberian Peninsula. Haplotype K has been exclusively identified in Black Baladi rabbit in Egypt was not detected in this study (Abdel-Kafy *et al*., 2018; Owuor *et al*., 2019; Ahmed *et al*, 2022).

Although rabbits from both clusters share the same breed names, their separation into two distinct haplotypes reflects differences in maternal lineages, as mitochondrial DNA is inherited exclusively through the maternal line. This suggests that rabbits of the same breed name in Central Java may have originated from multiple, independent introductions of European rabbits, each carrying different maternal haplotypes. These genetic differences may persist even if the animals are phenotypically similar, since selective breeding for production traits (e.g., growth rate, fertility, carcass quality) can mask underlying mitochondrial lineage variation. Nevertheless, differences in mitochondrial haplotypes can sometimes influence traits linked to energy metabolism, reproductive efficiency, or adaptability to local environments (John and Tsai, 2008; Kong et al., 2020). These findings suggest that Indonesian local Rabbit, Hycole, Hyla, and New Zealand White rabbit breeds in Central Java are descendants of European rabbits, but represent at least two distinct maternal lineages.

CONCLUSION

The Indonesian Local rabbit, Hycole, Hyla, and New Zealand White breeds in Central Java exhibit low genetic diversity and close genetic relationships. Conservation efforts are necessary to preserve genetic variation and ensure the sustainability of these breeds.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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