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Genetic variability analysis of indigenous Indonesian chickens and Hy-Line Brown using mitochondrial DNA D-Loop HV1 region

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Abstract. *Albani OP, Ismoyowati, Widodo HS, Rahayu S, Purwantini DD. 2025. Genetic variability analysis of indigenous Indonesian chickens and Hy-Line Brown using mitochondrial DNA D-Loop HV1 region. Biodiversitas 26: 2540-2547.* Genetic diversity plays a very significant role in the context of sustainable farming and food security. Indonesia is known for its diverse local chicken populations, whose genetic profile reflects their adaptation to the environments in which they live, as well as the various natural selection and breeding factors that differ from one region to another. Therefore, this research was carried out with the aim of knowing the genetic diversity and evolutionary origin of three Indonesian native chickens, namely Kedu Merah, Kampung, and Sentul chicken, based on mtDNA D-Loop sequences and compared it with a commercial stock of Hy-Line Brown laying hens. A total of 160 chickens, consisting of 40 chickens from each population, are analyzed using the PCR-SSCP technique, with the polymorphic results further sequenced. The result showed there are 9 haplotypes distributed across populations, with the Kedu Merah being the most diverse population (He = 0.624) and Hy-Line Brown being the least diverse (He = 0.219). Sequencing revealed that there are at least 19 polymorphic sites distributed along 167 bp of the HV1 region of the D-loop mtDNA sequence. Evolutionary analysis was performed by comparing the sequenced DNA with references from each chicken clade, and the result showed that Kedu Merah, Kampung, and Sentul chicken has a single maternal origin and belong to clade E, which can be traced back to the Indian subcontinent.

Keywords: Haplogroup, Hypervariable region 1 (HV1), Indonesian chickens, mitochondrial DNA, phylogenetic

Abbreviations: D-Loop mtDNA: Displacement Loop of mitochondrial DNA; He: Expected Heterozygosity; PCR-SSCP: Polymerase Chain Reaction-Single Strand Conformation Polymorphism

INTRODUCTION

The domestication of chickens is estimated to have begun around 8,000 years ago in Southeast Asia, with the red jungle fowl being the main ancestor of modern chickens. The domestication process continued, and the species spread to China and India about 3,000 years later (Hata et al. 2021). Numerous studies indicate that chickens may have multiple origins, resulting from domestication processes in diverse regions such as India, Southwest China, and Southeast Asia (Liu et al. 2006; Eda 2021; Peters et al. 2022; Gjøen and Jensen 2024; Zhao et al. 2024). Chickens were able to spread widely and quickly because of their ability to supply human needs for food at that time. Over time, the domestication process continued into selection.

Local chickens are characterized as those that have lived, adapted, developed, and reproduced within a specific region over an extended period (Irwanto et al. 2023). Although current livestock production heavily relies on commercial poultry, this does not mean that local poultry can be overlooked. Local chickens play a very important role ecologically, historically, and socio-culturally (Antikasari et al. 2023). Indonesia has at least 31 types of local chickens spread throughout its regions (Sulandari et al. 2008). This high diversity is a potential for Indonesia to develop superior local strains that can compete with commercial chickens. Some of the local Indonesian chickens, such as Kedu Merah, Kampung, and Sentul chickens, have high potential to be developed into superior strains (Ismoyowati et al. 2022). Kedu Merah, Kampung, and Sentul chickens are known to have high genetic diversity and good reproductive performance (Ismoyowati et al. 2010, 2012).

The development of superior strains often requires complete information about the genetic profile and its parental history. Analysis of the Displacement loop region of mitochondrial DNA (D-loop mtDNA) is one of the widely used techniques because, in addition to mapping genetic diversity, mtDNA can also provide insights into the evolutionary history of a population. The D-loop region is a crucial component of mtDNA, as it regulates the processes of replication and transcription within this genetic material (Saha et al. 2021). The D-loop is approximately 7% of the total length of the mitochondrial DNA genome (Pham et al. 2023). This region is also known to have the highest variation rate, often referred to as the Hypervariable region (HV region) (Boudali et al. 2022). The D-loop region is a non-coding sequence with a rapid evolutionary rate that serves as a valuable evolutionary marker. Notably, the

evolutionary rate of mtDNA is approximately 5 to 10 times faster than that of the nuclear genome (Guo et al. 2023). This is due to the accumulation of mutations caused by inefficient proofreading and repairing mechanisms (Carvalho et al. 2022). Moreover, mtDNA follows a maternal inheritance pattern, which prevents recombination and thereby provides clarity in tracing an individual's lineage (Al-Jumaili and Hanotte 2023). Mitochondrial DNA serves as an invaluable marker for reconstructing historical events and uncovering evolutionary connections between populations (Ran et al. 2023).

According to Ibrahim et al. (2023), cytochrome b gene sequences derived from mitochondrial DNA serve as effective tools for haplogroup identification and for identifying genetic relationships among sheep populations. Another research was done by Shi et al. (2025), by using genomic DNA and mitochondrial DNA sequences, they were able to uncover that adaptive introgression, gene regulation, and selection have shaped the genetic makeup of African cattle. Meanwhile, several studies using mtDNA have successfully revealed the maternal origins of local chicken species in various parts of the world. Ran et al. (2023) found that Meihuan chicken of China is distributed in four clades, A, B, E, and G. Vietnamese indigenous chickens appear to be more diverse, with distribution across 5 clades, A, B, C, D, and E (Nguyen et al. 2022). Meanwhile, Ren et al. (2023) found that Cambodian indigenous strains have very wide distribution in clades A, B, C, D, E, F, and J, with the most common found in clade D (44,4%). These research results further emphasize that Southeast Asian local chickens have many maternal origins. Given these successes, research is needed to map the genetic diversity and determine the position of Indonesian local chickens in the global evolutionary lineage.

MATERIALS AND METHODS

Materials and study area

The research was conducted in accordance with the procedures in accordance with the research ethics commission, Institute for Research and Community Service, Jenderal Soedirman University (No.709/UN23/ PT.01.02/2024). This research used 160 female chickens aged 36 weeks (9 months), consisting of Kedu Merah, Kampung, Sentul, and Hy-Line Brown commercial hens, raised on local chicken farms in Dukuhwaluh Village and at the Experimental Farm of the Faculty of Animal Husbandry, Universitas Jenderal Soedirman. The materials included chicken blood samples, FavorPrepTM Blood/ Cultured Cells Genomic DNA Extraction Mini Kit, PCR mix, one pair of primers, TBE 10× buffer, absolute ethanol, agarose, acrylamide, ammonium persulfate, TEMED, 8% glycerol, ethidium bromide, and DNA Ladder. The equipment used included disposable syringes, ice flasks, vacutainer tubes, micropipettes, centrifuges, water baths, collection tubes, PCR machines, a PCR kit, measuring glasses, submarine electrophoresis, gel casting glass, comb, and UV visualization.

Procedures

Blood collection and DNA isolation

A total of 3 mL of blood was taken from the subcutaneous brachial vein area of each chicken (total n = 160 samples), then placed into vacutainer tubes containing EDTA. Next, 50 µL was taken and placed into 1.5 mL Eppendorf tubes, then stored in a refrigerator. DNA isolation was performed following the procedures of the FavorPrepTM Blood/Cultured Cells Genomic DNA Extraction Mini Kit with some modifications.

Primer design

To amplify the Hypervariable region 1 (HV1) of the mtDNA D-loop sequence, specific primers are constructed based on published mtDNA sequences. The primer design was conducted on the NCBI Blast website. The primers are A-01397_HV1_(mtDNA)F 5'-TCTATATTCCACATTTCTC-3' OD 4.7 MW 5,663.8 GC 31% as the forward primer and A-01397_HV1(mtDNA)_R 5'-GCGAGCATAACCAAATGG-3' OD 5.4 MW 5,541.6 GC 50% as the reverse primer. The primers were designed to cut the sequence at bases 167 to 391 of D-Loop mtDNA, leaving approximately 225 nucleotides as the PCR product. The gene sequences are based on the partial mitochondrial genome of chickens from Komiyama et al. (2003) (GenBank accession number AB098668).

DNA amplification

The PCR reaction was carried out in a volume of 62.5 µL containing 31.25 µL PCR Mix, 2.5 µL primer for each forward and reverse (concentration 10 pmol/µL), 21.25 µL Nuclease Free Water, and 5 µL DNA sample. The PCR reaction was performed in 0.2 mL tubes, and the reaction was carried out on the Gene Amp*PCR system 9700 thermocycler (Applied Biosystem, USA). The PCR reaction used a pre-denaturation step at 92°C for 5 minutes, followed by 35 cycles with denaturation at 92°C for 30 seconds, annealing at 58°C for 30 seconds, and elongation at 72°C for 30 seconds. For the final cycle, the temperature was set to 72°C for 1 minute.

SSCP and sequencing

In the electrophoresis for Single Strand Conformation Polymorphism (SSCP), 12.5 µL of PCR product was mixed with 16 µL of loading buffer (95% dimethylformamide and 5% glycerol), then denatured at 98°C for 10 minutes. After that, the samples were placed in the freezer for 5 minutes, then electrophoresed for 12 hours in a 12% acrylamide gel (3.2 mL), 85% glycerol (0.47 mL), TBE 10× (0.8 mL), distilled water (3.442 mL), 10% APS (0.1 mL), and TEMED (0.01 mL). Electrophoresis was performed using a voltage setting of 300 V, a current of 15 mA, and a power of 1 W for 12 hours. Ethidium Bromide staining was used to visualize the bands formed. DNA sequencing in this study used the Sanger method and was tested at the *Laboratorium Penelitian dan Pengujian Terpadu* (LPPT) at Universitas Gadjah Mada, Yogyakarta, Indonesia.

Data analysis

Allele and genotype frequency was calculated using the following equation:

Allele frequency:

$$xi = \frac{2nii + \sum nij}{2N}$$

Genotype frequency:

$$xij = \frac{nii}{N} x100\%$$

Where:

xi : Allele frequency of ii
nii : Total individuals with ii genotype
nij : Total individuals with ij genotype
N : Total sample

Expected heterozygosity (He) is calculated using the following equation:

$$He = 1 - \sum_{i=1}^{n} p_i^2$$

Where:

pi : Frequency of the i-th allele
n : Number of alleles at a given locus

Haplotype diversity (Hd) and nucleotide diversity (π) are calculated with DnaSP using the following formula:

Haplotype diversity:

$$Hd = \frac{n}{n-1}\left(1 - \sum_{i=1}^{k} p_i^2\right)$$

Nucleotide diversity:

$$\pi = \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \frac{\pi_{ij}}{n(n-1)/2}$$

Where:

Hd : Haplotype diversity index
n : Total number of individuals in a sample
k : Sum of different haplotypes
pi : i-th haplotype frequency in population
π : Nucleotide diversity index
n : Number of sequences
πij : Proportion of nucleotide sites when sequences i and j differ

The sequences obtained were analyzed using various types of computer software based on methods from the publications of Sulandari et al. (2008), Kawabe et al. (2014), and Godinez et al. (2019). MEGA 11 was used to trim and align the obtained sequences. The alignment of D-loop sequences was performed using the reference sequence from GenBank accession number AB0986688 (Komiyama et al. 2003), as well as representative sequences of each clade from the references Liu et al. (2006) and Oka et al. (2007). Phylogenetic relationships were reconstructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method and the Jukes-Cantor parameter. Polymorphic site determination, haplotype diversity calculation, nucleotide diversity calculation, and Analysis of Molecular Variance (AMOVA) were performed using DnaSP6 software. Median-joining analysis was conducted using the NETWORK 10200 application.

RESULTS AND DISCUSSION

SSCP analysis result

Most of the genotypes that appear are homozygous (Figure 1). This is possible because the sequences used come from mitochondrial DNA, which has a maternal inheritance pattern. According to Jeedigunta et al. (2021), mtDNA inheritance involves active mechanisms of degradation and dilution that effectively remove paternal mtDNA during fertilization, ensuring that only maternal mtDNA is passed on to the offspring. This mechanism naturally increases the likelihood of homozygous genotypes.

Based on the calculation of genotype frequency, haplotype 8 with genotype GG was found to be the most common genotype, with a frequency value of 18.8% (Table 1). This indicates that in a random chicken population consisting of Kedu Merah, Kampung, Sentul, and Hy-Line Brown hens, the Hy-Line Brown hens with genotype GG will appear with a percentage of 18.8%. These results align with the theory that laying hens are the result of a selection process and have been bred to have a more uniform genetic profile compared to local chickens (Elferink et al. 2012).

Furthermore, on molecular parameters, it is known that the nine haplotypes detected in this study consist of 8 different alleles (Table 2). This indicates that the loci on mitochondrial DNA are multiallelic. This result is in line with the statement by Boudali et al. (2022) that the hypervariable region of mitochondrial DNA is the region with the highest variation compared to other regions or even the nuclear genome. Other studies conducted by Okani-Onyejiaka et al. (2022) on Nigerian local chickens also support the results of this study, where they also found that the loci in mitochondrial DNA are indeed multiallelic.

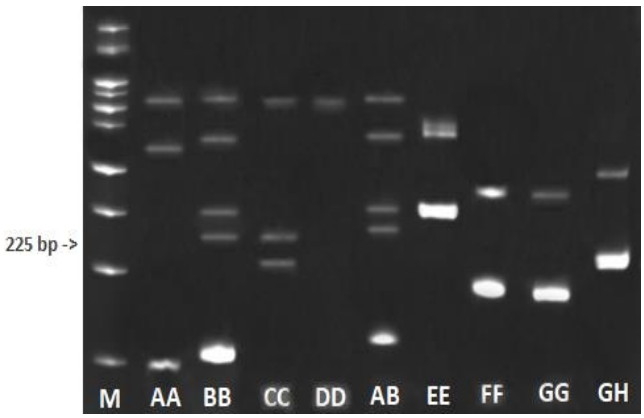

Figure 1. Identified genotypic band patterns based on the PCR-SSCP electrophoresis result. M: DNA marker

In accordance with the previous genotype calculations, the highest allele frequency was found in the G allele of Hy-Line Brown hens, which was 21.9% (Table 2). As for the proportion of alleles in each strain, none exceeded 75% except for the G allele of Hy-Line Brown hens. Although there is currently no consensus on the threshold for genotype frequency or allele frequency to be considered uniform, it can be said in this study that the proportion of the G allele exceeding 75% indicates that the Hy-Line Brown hen population has more uniform individuals than the local chicken population. As for the proportion of alleles in the local strains of Kedu Merah, Kampung, and Sentul, all were below 75%, meaning these populations still have moderate/high diversity.

According to the definition by Kanaka et al. (2023), heterozygosity is the probability that two alleles randomly selected at a particular locus are different from each other. Therefore, it can be concluded that the higher the heterozygosity value, the higher the genetic diversity in the population. Conversely, the lower the heterozygosity value, the lower the genetic diversity in the population. According to Febrianto et al. (2018), heterozygosity can be classified as medium if it is in the range (He = 0.278-0.499). Based on this, the Kedu Merah strain population has the highest genetic diversity. At the same time, the Kampung and Sentul chickens fall into the category of medium genetic diversity, and the Hy-Line Brown hens have low genetic diversity (Table 3). The high heterozygosity value is influenced by allele frequency and the number of alleles that appear at that locus. In this case, the Kedu Merah chickens have 3 haplotypes with relatively low allele frequencies. High heterozygosity values also indicate that the population has good adaptability and survival prospects (Kossoga et al. 2023). Conversely, the Hy-Line Brown hen population has low heterozygosity due to selection pressure on commercial chickens, which results in low genetic diversity.

Sequence analysis result

Visual observation of SSCP electrophoresis results showed that 9 haplotypes were present in all 160 samples, so sequencing in this study was only conducted on representative samples from each haplotype. A total of 11 samples, consisting of three Kedu Merah chicken samples, three Kampung chicken samples, three Sentul chicken samples, and two Hy-Line Brown hen samples, were sent for sequencing. However, only 10 samples met the criteria and could be sequenced. Following the trimming process and sequence quality assessment, only 167 base pairs from the original 225 bp target amplicon were deemed suitable for further analysis.

Polymorphism analysis results using DNASP software showed that at least 19 polymorphic sites were identified (Figure 2). The discovery of polymorphic sites in the sequences indicates that the mitochondrial DNA of chickens is actively evolving. From the polymorphism analysis, we can see that the mutations occurring, which is only involve substitutions, and no insertions or deletions were found.

According to Garcia et al. (2021), a value of 1 for haplotype diversity means that each individual in a population has a different haplotype. Referring to the results of Teinlek et al. (2018), haplotype diversity in the range of (0.45 to 0.89) is classified as medium. Therefore, the populations of Kedu Merah, Kampung, and Sentul chickens in this study fall into the medium diversity category. In comparison, the Hy-Line Brown hen population falls into the low diversity category (Table 4).

Table 1. Genotype frequency of each chicken strain

Strain	Haplotype	Genotype	Individuals	Genotype frequency
Kedu Merah	Haplotype 1	AA	9	5.6%
	Haplotype 2	BB	20	12.5%
	Haplotype 3	CC	11	6.9%
Kampung	Haplotype 4	DD	27	16.9%
	Haplotype 5	AB	13	8.1%
Sentul	Haplotype 6	EE	26	16.3%
	Haplotype 7	FF	14	8.8%
Hy-Line	Haplotype 8	GG	30	18.8%
Brown hens	Haplotype 9	GH	10	6.3%
Total			160	1

Table 3. Expected heterozygosity (He) of each chicken strain

Strain	Expected heterozygosity
Kedu Merah	0.624
Kampung	0.492
Sentul	0.455
Hy-Line Brown hens	0.219

Table 2. Frequency and proportion of each allele

Allele	Strain				Proportion	Frequency
	Kedu Merah	Kampung	Sentul	Hy-Line Brown hens		
A	22.5%	16.3%	0	0	38.8%	9.7%
B	50%	16.3%	0	0	66.3%	16.6%
C	27.5%	0	0	0	27.5%	6.9%
D	0	67.5%	0	0	67.5%	16.9%
E	0	0	65%	0	65%	16.3%
F	0	0	35%	0	35%	8.8%
G	0	0	0	87.5%	87.5%	21.9%
H	0	0	0	12.5%	12.5%	3.1%
Total	1	1	1	1	4	1

The highest nucleotide diversity was found in the Kedu Merah population, where π was 0.0319 (Table 4). This indicates that in the 167 bases that have been sequenced, there will be an average of about 3.19% nucleotide difference at each site between two sequences in the population. On the other hand, the population with the lowest nucleotide diversity was found in Kampung chickens. These results contradict the findings of Saelao et al. (2020), who compared nucleotide diversity in wild and commercial bee populations. Saelao et al. (2020) found that nucleotide diversity in commercial populations almost always had lower values because commercial populations have been selectively bred to have uniform characteristics. Such selection pressure undoubtedly affects by lowering nucleotide diversity in the population (Booker and Keightley 2018). This discrepancy can be explained by the use of mitochondrial DNA as the object of this study, unlike the studies of Booker and Keightley (2018) and Saelao et al. (2020) that used genomic DNA. Mitochondrial DNA is a conserved non-coding sequence that is inherited without recombination, so breeding and selection processes presumably do not significantly bias the nucleotide diversity index of mtDNA. In addition, all Tajima values are positive, indicating that every population is experiencing balancing selection (Nayak et al. 2024). Hy-Line Brown hens show the highest Tajima's D, suggesting a stronger balancing selection since it is commercially bred. Meanwhile, the positive value of the indigenous breeds might indicate that the population is undergoing a selection process, which is potentially due to natural environment adaptation or human-driven breeding strategies (Mengistie et al. 2023).

The results of the Analysis of Molecular Variance (AMOVA) of mitochondrial DNA sequences from the four chicken populations showed that 87.60% of the recorded genetic variation was within populations, while the remaining 12.40% was between populations (Table 5). The high percentage of within-population variation (87.60%) indicates the richness of genetic diversity within each population. FST value of 0.12403 shows that there is a moderate level of genetic differentiation between populations, which further implies that each population may exhibit its own unique traits. This diversity is important because the higher the variation between populations, the higher the chances for a population to adapt to its environment (Nonić and Šijačić-Nikolić 2021). DeWoody et al. (2021) also stated that genetic diversity is very important for the resilience of a population because it provides a broad genetic basis for organisms to respond to environmental changes and selective pressures.

Phylogenetic relationship reconstruction and median joining analysis were conducted to determine the evolutionary origins of local strains in this study. Referring to several studies such as Liu et al. (2006), Oka et al. (2007), Kawabe et al. (2014), and Okani-Onyejiaka et al. (2022), 11 mtDNA sequences were obtained, each representing a group/clade of chicken origins. One reference sequence from Komiyama et al. (2003) was also used as a non-cluster comparator. The list of sequences used in this study, along with their accession numbers, is recorded in Table 6.

	0	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	1
	0	2	2	4	4	5	6	7	8	8	9	0	1	1	1	3	4	1
	1	0	4	5	6	9	9	4	9	2	4	9	5	9	2	3	8	9	3	5	9	2
REF	.	T	C	A	T	T	T	C	T	T	A	T	C	T	T	C	T	A	C	A	.
KD11	.	.	T	.	C	C	C	T	A	G	G	C	.	C	C	T	C	C	.	.	.
KD23	.	.	.	.	C	C	C	T	.	.	G	C	.	C	.	T	C	.	.	.	.
KD43	.	C	.	.	C	C	C	T	.	.	G	C	.	.	.	T	C	.	.	G	.
KP4	.	.	.	.	C	C	C	T	.	.	G	C	T	C	C	T	C	.	.	.	.
KP24	.	.	T	.	C	C	C	T	.	.	G	C	.	C	C	T	C	.	.	.	.
S2	.	.	.	.	C	C	C	T	.	.	G	C	.	C	C	T	C	.	.	.	.
S19	.	.	.	.	C	C	C	T	.	.	G	C	.	C	C	T	C	.	.	.	.
S28	.	C	.	.	C	C	C	T	A	.	.	C	.	.	.	T	C	.	.	.	.
P5	.	C	.	.	C	C	C	T	.	.	.	C	.	.	.	T	C	.	.	G	.
P13	.	C	.	G	C	C	C	T	.	.	.	C	.	.	.	T	C	.	T	.	.

Figure 2. Polymorphic sites detected and their relative position to the D-loop sequence. Vertically oriented numbers indicate the site position, and the sequences shown are only the variable sites. Dots (.) indicate similarity with the Reference sequence (REF)

Table 5. Analysis of Molecular Variance (AMOVA) results of 4 chicken populations

Source of variation	Percentage of variation
Between population	12.40%
Within population	87.60%
Total	100%
FST: 0.12403	

Table 4. Haplotype and nucleotide diversity of each chicken strain.

Strain	n	Polymorphic sites/Total number of mutations	Hn	Hd	II	Tajima D
Kedu Merah	40	16	3	0.68	0.03194	1.28
Kampung	40	12	2	0.45	0.0120	1.89
Sentul	40	12	2	0.47	0.0200	1.22
Hy-Line Brown hens	40	11	2	0.39	0.0180	2.01

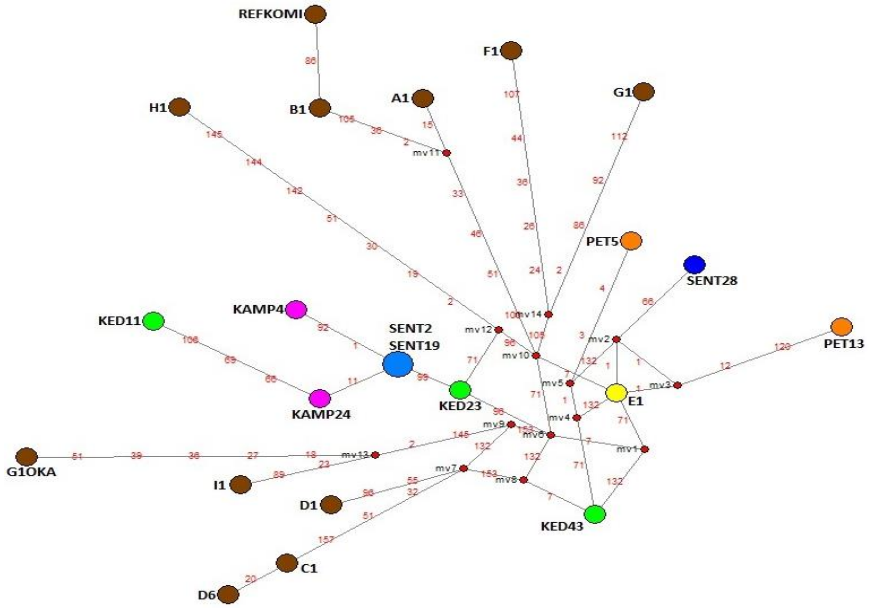

Figure 4. Median-joining network profile of mtDNA D-Loop haplotypes observed among samples and references. The circle size corresponds to haplotype frequency, and the numbers on the line correspond to mutational positions connecting haplotypes. Red circles are median vectors used in connecting indirectly related haplotypes. REFKOMI: Non-clade reference; A1-G1OKA: Clade references; KED: Kedu Merah; KAMP: Kampung; SENT: Sentul; PET: Hy-Line Brown hen

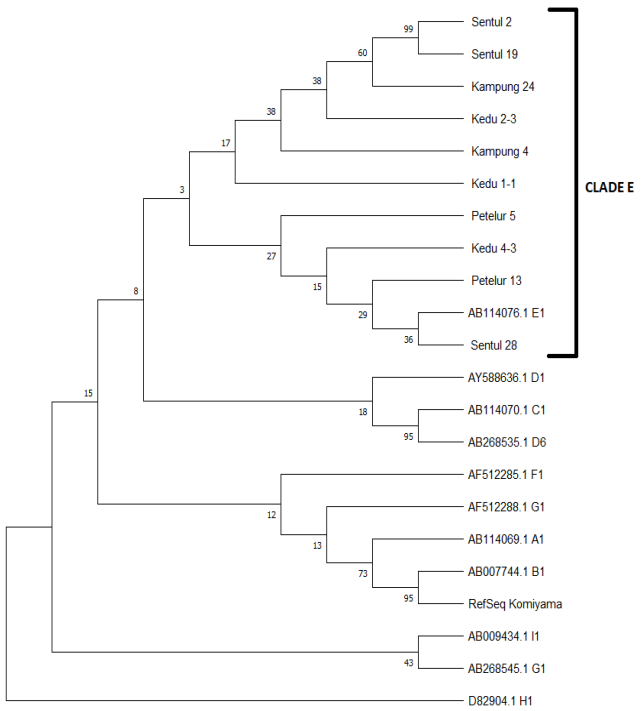

Figure 3. UPGMA tree showing the evolutionary relationship between sample populations and haplotypes of reference based on the mtDNA D-Loop region. The numbers at nodes represent the percentage bootstrap values for interior branches after 1500 replications

Looking at the structure of the phylogenetic tree that was formed, it can be said that all the chicken samples used in this study have genetic proximity to the E1 reference sequence from Liu et al. (2006). Besides the E1 reference, there are no other reference sequences that have a close relation to the test sequences in this study. Therefore, it can be concluded that the Kedu Merah, Kampung, Sentul, and even Hy-Line Brown hen belongs to clade E (Figure 3). This indicates that all the chicken strains used in this study come from a single matrilineal line. Contrary to previous assumptions that local chickens in Indonesia evolved from more than one ancestor, the phylogenetic analysis results of this study show that the local Kedu Merah, Kampung, and *Sentul* strains originated from a single ancestor from clade E. According to Liu et al. (2006), clade E is one of the two chicken clades with the widest distribution in the world, covering Europe, the Indian subcontinent, China, and Southeast Asia. Clade E is also believed to have originated from the Indian subcontinent.

The mono-matrilineal distribution of the Kedu Merah, Kampung, and Sentul chicken into clade E offers a new theory that clade E originated from the Indian subcontinent. Historically, Indonesia once had a Hindu-Buddhist kingdom era where most of the culture at that time was influenced by Indian culture. According to Pathak (2024), the spread of Hinduism and Buddhism in Indonesia occurred through trade and cultural exchange with India, which happened around the first century AD. It is possible that one of the commodities traded at that time was chickens. The Indian people brought chickens from their region to Indonesia, causing the local chickens that live today to carry genetic traces back to their origin.

The results of the median-joining network analysis of the nine haplotypes can be seen in Figure 4. As with the results of the phylogenetic tree reconstruction in Figure 5.3, the median-joining analysis in this study also shows that the 9 haplotypes have genetic proximity and lead to haplotype E1 (yellow circle). This indicates that each haplotype in this study shares a common ancestor with haplotype E1, which is estimated to originate from the Indian subcontinent.

Table 6. Haplotypes of reference obtained from GenBank

Haplotype	GenBank accession number	Reference
Haplotype 1-9		This research
REFKOMI	AB098668	Komiyama et al. (2003)
Liu_A1	AB114069	Liu et al. (2006) haplotype A1
Liu_B1	AB007744	Liu et al. (2006) haplotype B1
Liu_C1	AB114070	Liu et al. (2006) haplotype C1
Liu_D1	AY588636	Liu et al. (2006) haplotype D1
Liu_E1	AB114076	Liu et al. (2006) haplotype E1
Liu_F1	AF512285	Liu et al. (2006) haplotype F1
Liu_G1	AF512288	Liu et al. (2006) haplotype G1
Liu_H1	D82904	Liu et al. (2006) haplotype H1
Liu_I1	AB009434	Liu et al. (2006) haplotype I1
Oka_D6	AB268535	Oka et al. (2007) haplotype D6
Oka_G1	AB268545	Oka et al. (2007) haplotype G1

The presence of median vectors branching into two or more haplotypes indicates the existence of ancestral haplotypes that are not/have not been recorded in this study. A star-like network pattern indicates that individuals undergo rapid and complex divergent evolution. According to the research of Alexander et al. (2015), mitochondrial DNA in chickens shows a very rapid divergent evolution pattern in a relatively short period. As a consequence, chickens may have very high mitochondrial DNA genetic diversity. This diversity can be seen from the number of polymorphic sites detected (Figure 2).

In conclusion, this research demonstrates the high genetic diversity of Indonesian native chickens, particularly when compared to the commercial stock population. The Kedu Merah population, among the three native strains, exhibits the highest diversity index. The evolutionary analysis and reconstruction, a significant part of this study, reveal that Kedu Merah, Kampung, and Sentul chickens share a single maternal origin and belong to clade E; this lineage can be traced back to the Indian subcontinent.

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