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Genetic Characterization of Sumatran Mirah Chicken Based on Mitochondrial D-loop Region Sequence

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ABSTRACT

Mirah chickens are Indonesian indigenous chickens that originate from Simalungun Regency, North Sumatera Province of Indonesia. The study aimed to determine the genetic characterization of Mirah chicken based on the mitochondrial D-loop region (838 bp). Twenty Mirah cocks from Simalungun Regency of Indonesia were used in the present study. The results showed that fourteen haplotypes were found in the studies of birds based on sixteen mutation sites. Therefore, these haplotype and nucleotide diversities in the partial D-loop region of the Mirah chicken were classified as high. The Median-joining tree revealed that the Mirah chickens were classified in a similar cluster with the Red jungle fowl (*Gallus gallus*). Ten haplotypes of birds were close to *G. g. bankiva*, and four haplotypes of birds were close to *G. g. gallus*. In conclusion, Mirah chickens had the genetic introgression from two sub-species of Red junglefowl, i.e., *G.g. gallus* and *G.g. bankiva*. Hence, the pure breeding program for Mirah chickens is important to conserve their genetic resources from extinction.

1. Introduction

Mirah chicken is one of the Indonesian native chickens from Sumatera Island, but it's not officially approved by the Indonesian Ministry of Agriculture. Generally, Mirah chickens were kept by the farmers at Simalungun Regency for meat and egg production. Mirah chicken was considered to be descendants of the red jungle fowl, which have been domesticated by the people of Simalungun Regency, so its phenotypic characteristics were similar to the red jungle fowl. The adult weight of the Mirah chicken was about 3.10 kg (male) and 2.10 kg (female), with the egg weight of about 35-37 g (Siagian *et al*. 2013). Previously, Mirah chickens were used for special culinary of Dayok Nabinatur for noblemans of Simalungun (Sumbayak *et al*. 2018) and also used for sacrificial animal in the tradition ceremony of Paabingkon Pahompu (Marbun 2023). Presently, the Dayok Nabinatur is one of the famous culinary traditional foods in Sumatra and has the potency for culinary tourism in Simalungun (Damanik and Sinaga 2023; Haloho 2023; Indra *et al*. 2024). As with the historical chickens, a study to determine the genetic characterization is important for the genetic conservation program in the future. Unfortunately, most of the Mirah chickens were kept by farmers with extensive traditional systems without good breeding practices. Hence, genetic introgression from other chicken breeds may occur in Mirah chickens through crossbreeding among them.

A mitochondrial DNA (mtDNA) Displacement loop (D-loop) gene was widely used for the genetic characterization of Indonesian native chickens (Sulandari *et al*. 2008; Sulandari and Zein 2009; Herrera *et al*. 2017). The D-loop gene is a regulatory region that is the starting site for mitochondrial DNA replication. Otherwise, the D-loop gene sequence can provide more information and unbiased data essential to characterize and conserve the genetic resources of

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animals (Islam *et al*. 2019). Despite this, the D-loop gene is a non-coding region of the mtDNA that evolves faster than other regions in the mitochondrial genome. Therefore, the rate of evolution in the D-loop region is five to ten times greater than the nuclear genome (Muchadeyi *et al*. 2008). Through studies of the maternally inherited D-loop gene sequence, it is possible to assess the genetic diversity, ancestry, and evolutionary history in the genetic resources of animals.

Recently, the government of Simulangun Regency decided to use a Mirah chicken as the animal mascot in Pematang Siantar City. Hence, Mirah chickens are one of the social culture elements for Simalungun society. Therefore, this study on the genetic characterization of Mirah chicken will significantly contribute to the national program of conservation, characterization, and further improvement of native chicken genetic resources.

2. Materials and Methods

2.1. Ethical Approve

The animal welfare of medical/health research was approved by the Health Research Ethical Committee of Medical Faculty Nommensen HKBP University (No. 469A/KEPK/FK/I/2023) characterization.

2.2. Sample and Research Site

Twenty (20) heads of mixed-sex Mirah chickens (4-6 months of age) were used in this study and collected from the Mirah chicken farms at Simalungun Regency, North Sumatera Province of Indonesia (Figure 1). This area is situated at latitude 02°36'-03°18' N and longitude 98°32'-99°35' S. It is placed at 0-1500 m asl with an air temperature of 26.9°C, relative humidity of 82.3 % and rainfall of 2,950 mm/year. An amount of 3 µL blood samples was collected from each bird through the brachial vein with venoject vacutainer tube containing EDTA. In addition, 30 Sequence references of the mitochondrial D-loop region of Indonesian chickens and sub-species of *G. gallus* were accessed from the NCBI database (Table 1).

2.3. PCR and Sequencing

The DNA extraction was performed using a Genomic DNA Extraction Kit (Geneaid, Taiwan) following the manufacturer protocols. The PCR reaction was performed in a total volume of 30 µL containing 9 µL of DNA template (±5.75 ng/µL); 1.5 µL of each primer (10 pmol); 15 µL of PCR mastermix (Bioline, USA) and 4.5 µL of nuclease-free water. A primer pairs of Forward: 5'-AGG ACT ACG GCT TGA AAA GC -3' and Reverse: 5'- CCA TAC ACG CAA ACC GTC TC -3' (Sulandari *et al*. 2008) was used to amplify the mitochondrial D-loop gene (GenBank: NC_001323.1) along 838 bp (Figure 2A). The amplification program was performed in a mastercycler gradient (Eppendorf, Germany) with pre-denaturation at 95°C for 5 s and followed by 35 cycles of denaturation at 95°C for 15 s; annealing at 60.4°C for 15 s; extension at 72°C for 30 s and final extension at 72°C for 3 min. The electrophoresis of PCR products was performed in 1% agarose gel containing the DNA staining dye (FluoroStainTM, Taiwan) and captured by G-box Documentation System (Syngene, UK). Therefore, the forward sequencing analysis was

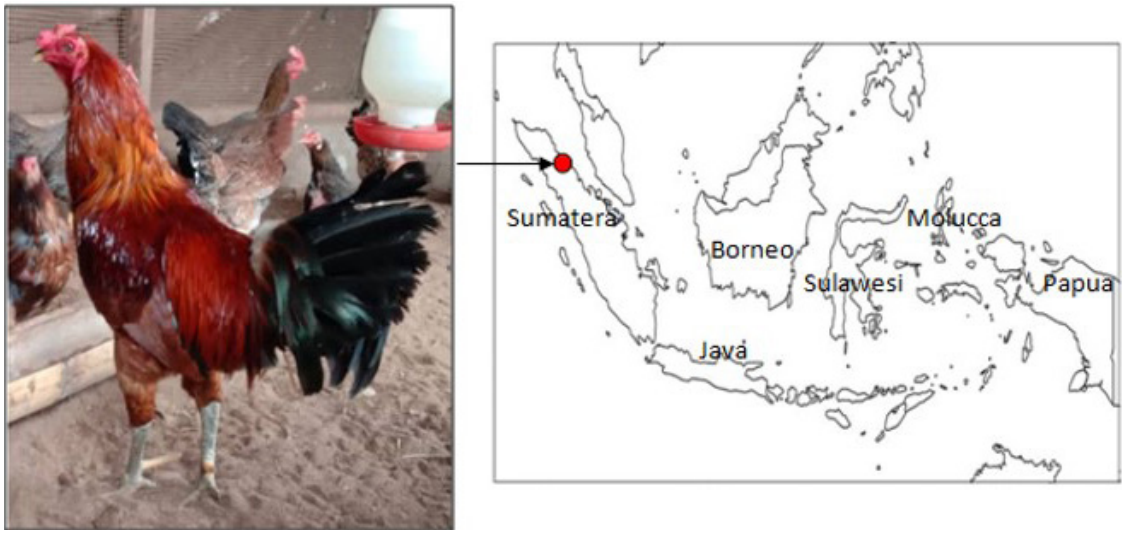

Figure 1. The breeding farm of Mirah chicken at Simalungun Regency, Sumatera island of Indonesia

Tabel 1. Sequence reference of mitochondrial D-loop region of Indonesian chickens and sub-species of *G. gallus* from NCBI database

GenBank	Species (breed)	Origin	References
KY039428	*G. gallus*	Sulawesi Island	
KY039424	*G. gallus*	New Guinea Island	
KY039427	*G. gallus*	Sulawesi Island	
KY039426	*G. gallus*	Lombok Island	
KY039425	*G. gallus*	Molucca Archipelago	
KY039422	*G. gallus*	Borneo Island	
KY039420	*G. gallus*	Java Island	
KY039418	*G. gallus*	Java Island	
KY039422	*G. gallus*	Borneo Island	
KY039419	*G. gallus*	Java Island	
KY039423	*G. gallus*	Papua Island	
KY039395	*G. gallus*	Molucca Archipelago	
KY039429	*G. gallus*	Sulawesi island	
NC001323	*G. gallus*	-	
NC007239	*G. lafayetti*	-	
NC007240	*G. sonneratii*	-	
NC007238	*G. varius*	-	
AP003323	*G. g. bankiva*	-	
OL988893	*G. g. murghi*	-	
GU261696	*G. g. jaboullei*	-	
AB007720	*G. g. gallus*	-	
OM634640	*G. g. spadiceus*	-	
KR536066	*G. g. domesticus* (Gaga)	Sulawesi Island	
KR536041	*G. g. domesticus* (Brugo)	Sumatra Island	
KR536027	*G. varius*×*G. g. domesticus* (Bekikuk)	Java Island	
KR536055	*G. g. domesticus* (Cemani)	Java Island	
KR536151	*G. g. domesticus* (Pelung)	Java Island	
KR536166	*G. g. domesticus* (Sumatera)	Sumatra Island	
KR536127	*G. g. domesticus* (Kampung)	Indonesia	
KR536135	*G. g. domesticus* (Nunukan)	Indonesia	

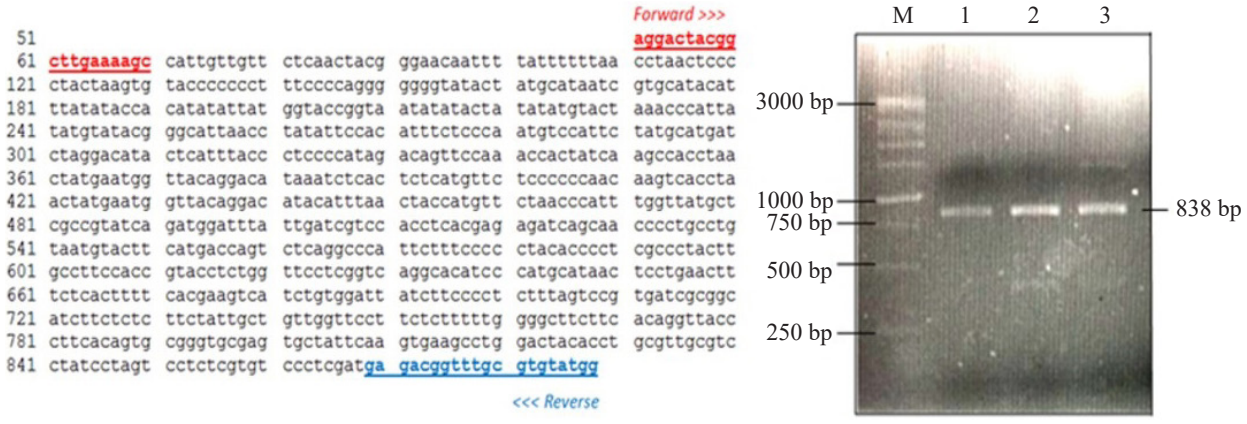

Figure 2. Target sequence of chicken mitochodrial D-loop gene (GenBank: NC_001323.1) based on the primer pairs (A) and amplification of mitochondrial D-loop gene (838 bp) on 1% agarose gel (B). M: DNA ladder 1 kb; Line 1-3: DNA sample

performed by 1st Base Laboratory Service (Malaysia).

2.4. Data analysis

The DNA sequence alignment was performed using the BioEdit package (Hall 2011). The phylogenetic tree of birds was constructed by the MEGA-X package (Hall 2013) with the UPGMA 1000×bootstrap replicates method. A DNAsp package (Librado and Rozas 2009) was used to estimate haplotype diversity (Hd), nucleotide diversity (pi), Fu's Fs statistics, and Tajima's D test. Therefore, a NETWORK package (Bandelt *et al.* 1999) was used to construct a median-joining network of birds under study.

3. Results

Along 838 bp of mitochondrial D-loop gene was successfully amplified on 1% agarose gel (Figure 2B). The haplotype diversity (Hd) in birds was 0.96 and categorized into a high category (Hd > 0.50), as shown in (Table 2). Nonetheless, the nucleotide diversity (pi) was in the low category (pi<0.01). In addition, the neutrality test of Fu's Fs statistics and Tajima's D test were a negative value. Therefore, sixteen mutation sites were found in the mitochondrial D-loop gene of birds reveal of fourteen haplotypes (Table 3). Generally, birds in the present study are classified into the H3 group (3 birds) and followed by H2, H7, and H9 groups with 2 birds per group. While other haplotypes consisted of 1 bird. According to the median-joining network, Mirah chickens can be characterized into two clusters (clades) of cluster A (5 birds) and cluster B (14 birds), as shown in (Figure 3A). The phylogenetic tree in several Indonesian chicken breeds revealed that most of the Indonesian chicken breeds were classified into one cluster with *G. g. bankiva* due to Mirah chickens with H2, H3, H4, H5, H6, H7, H10 and H11 haplotypes (Figure 3B). Interestingly, four haplotype groups in the Mirah chicken (H1, H8, H9, and H12) were classified in a separate cluster with *G. gallus* domesticus from Nunukan of Indonesia (KR536135).

Table 2. Genetic diversity in the partial D-loop gene of Mirah chicken

Parameter	Value
Haplotype diversity (Hd)	0.96±0.03
Nucleotide diversity (pi)	0.01±0.001
Fu's Fs statistic	-6.76
Tajima's D test	-0.37

In addition, no Mirah chicken was classified into similar clusters with *G.g. murghi* and *G. g. jabouillei.* According to the D-loop gene diversity, Mirah chickens are composed of two distinct matrilineal lineages, i.e., *G. g. gallus* (Gallus clade) and *G .g. bankiva* (Bankiva clade). Despite this, two native chickens of Indonesia (KR536127 and KY039429) are grouped in a similar cluster with *G. g. murghi* and *G. g. jabouillei.*

4. Discussion

In this study, the mtDNA D-loop gene could discriminate Mirah chicken into two common clades. Previous studies reported that the mtDNA D-loop gene was also able to determine many clades in native chicken breeds from Nigeria (2 clades), Lao (5 clades), Russia (3 clades), China (4 clades), Thailand (6 clades), Bangladesh (4 clades), Samar island of Philippines (4 clades), Korea (2 clades), Vietnam (5 clades), Cambodia (7 clades) and Nigeria (2 clades) populations (Wani *et al*. 2014; Kawabe *et al*. 2014; Dyomin *et al*. 2016; Gao *et al*. 2017; Teinlek *et al*. 2018; Islam *et al*. 2019; Godinez *et al*. 2019; Kim and Yoon 2020; Nguyen *et al*. 2022; Ren *et al*. 2022; Okani-Onyejiaka *et al*. 2022). Contrastly, low genetic diversity of mtDNA D-loop gene was observed in Liberian native chickens (Tor *et al*. 2021). High number of chickens clade indicating high genetic diversity in chickens population.

The haplotype diversity (Hd) and nucleotide diversity (pi) in Mirah chicken were classified into high and low categories, respectively (Tabel 2). According to Bandelt *et al*. (1999), the Hd value less than 0.50 represents low genetic diversity. While the Hd value of more than 0.50 represents high genetic variation. In

Table 3. Detection of mutation sites in partial D-loop gene of Mirah chicken

Haplotype (H)	Position*																
	N	196	214	219	235	278	303	306	327	339	352	396	423	425	443	579	683
H1	1	T	C	A	G	T	T	T	C	G	T	G	G	G	T	G	A
H2	2	T	T	A	G	C	C	C	C	A	T	G	G	G	C	G	G
H3	3	T	T	A	G	C	C	T	C	A	T	A	G	G	C	G	G
H4	1	T	T	A	G	C	C	T	C	G	T	G	G	G	C	G	G
H5	1	T	T	A	A	C	C	T	C	A	T	G	G	G	C	A	G
H6	1	T	T	A	G	C	C	T	C	A	T	A	A	G	C	G	G
H7	2	T	T	A	G	C	C	T	C	A	T	A	A	A	C	G	G
H8	1	T	C	A	G	T	T	T	C	A	T	A	G	G	T	G	G
H9	2	C	C	A	G	T	T	T	C	A	T	G	G	G	T	G	G
H10	1	T	T	A	G	C	C	T	C	A	C	A	G	G	C	G	G
H11	1	T	T	A	G	C	C	T	C	A	C	G	G	G	C	G	G
H12	1	T	C	G	G	T	T	T	T	A	T	G	G	G	T	G	G
H13	1	T	T	A	A	C	C	T	C	A	T	A	A	G	C	G	G
H14	1	T	T	A	G	C	C	T	C	A	T	G	G	G	C	G	G

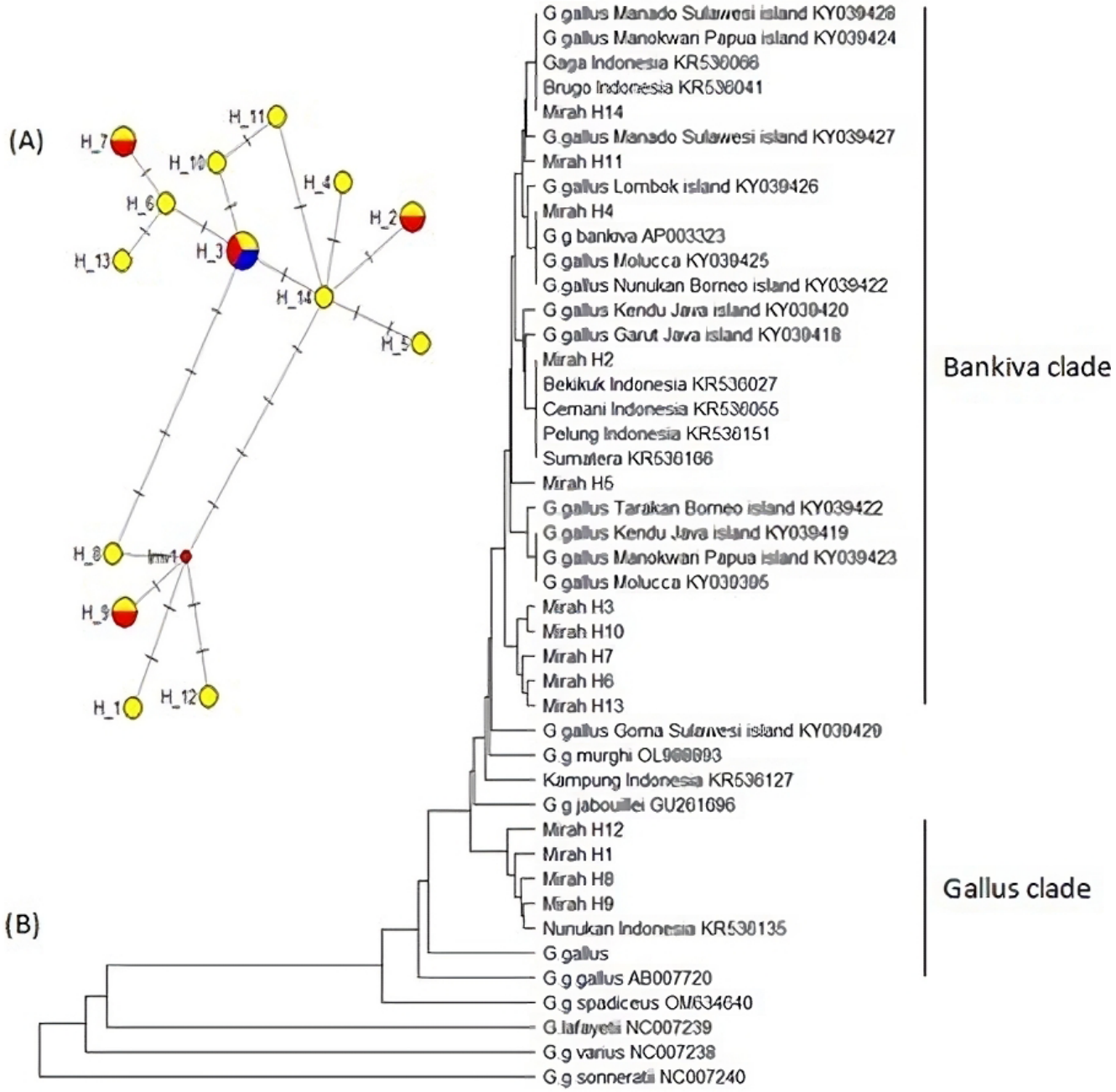

Figure 3. Median-joining network among D-loop haplotype of Mirah chicken (A) and UPGMA-tree (1000×bootstrap replications) of Mirah chicken and many Indonesian chicken breeds (B). Different colors in the median-joining network represent the chicken number

addition, the pi value is classified into low (0.01-0.04), moderate (0.05-0.07), and high (0.08-1.00) categories. Many previous studies reported the close Hd/pi value in native chicken breeds from Tibet (0.92/0.02), Bangladesh (0.92/0.01), Samar island of Philippines (0.92/0.01), Korea (0.92/0.06), China (0.92/0.01) and Vietnam (0.96/0.01) based on D-loop gene diversity (Zhang *et al*. 2017; Islam *et al.* 2019; Godinez *et al.* 2019; Kim *et al*. 2020; Huang *et al*. 2021; Nguyen *et al*. 2022).

According to the mtDNA D-loop gene sequence, Mirah chicken had two different Bankiva and Gallus clades lineages. There are four sub-species of Red junglefowl (*G. gallus*) such as: *G. g. spadiceus*; *G. g. gallus*; *G. g. jabouillei*; *G. g. bankiva* and *G. g. murghi* that are distributed across Southeast Asia (Hata *et al*. 2021). The *G. g. spadiceus* is distributed across southwestern China, northern Thailand, and Myanmar. Thus, *G. g. gallus* is distributed across northern Sumatera, the Malayan peninsula, and Indo-China

regions. Therefore, *G. g. jabouillei* is confined to the South China and North Vietnam regions. In addition, *G. g. bankiva* is distributed across southern Sumatera and western Java regions. Subsequently, *G, g, murghi* is distributed across the northern Indian subcontinent (Eda 2021).

Most of the Mirah chickens were close to *G. g. bankiva* and similar to the other Indonesian chickens. However, many Mirah chickens are close to *G. g. gallus*. Sumatra island has two sub-species of *G. g. gallus* and *G. g. bankiva*. Hence, the genetic introgression in both sub-species can occur in the wild. Interestingly, there is no genetic introgression between both species represented in Mirah chickens. Generally, the mtDNA D-loop gene revealed that *G. gallus* is the original ancestor in native chickens of Iran (Mousavizadeh and Nassiry 2014) and Liberia (Tor *et al*. 2021). Meanwhile, *G. g. spadiceus* is the origin ancestor of native chickens of Thailand (Hata *et al*. 2021), and *G. g. murghi* is the origin ancestor of native chickens of Bangladesh and Russia (Islam and Nishibori 2012; Oyun *et al*. 2015).

The haplotype diversity in the Fs statistics and Tajima's D test showed negative values, indicating species expansion. Hence, genetic conservation in Mirah chickens is important to prevent genetic drift, which had a negative effect on the population. A breeding program that avoids mating between individuals with close genetic relationships is important to increase survival rates in the animal population (Han et *al*. 2023).

However, the early investigation in the present study revealed that Mirah chickens consist of two different matrilineal lineages. It is indicated that the genetic conservation program for this chicken is important for protecting its genetic resources. Currently, selected native chickens are increasingly raised in North Sumatra Province, such as KUB, Bangkok, Joper, Mardi, and others, so that the culinary culture of serving Dayok Nabinatur with Mirah chicken might be replaced by other types of native chicken (Sitanggang 2015; Hasyim *et al*. 2021; Jailani and Ginting 2024). The government must develop the villager breeding center (VBC) at the breeding tracts of Mirah chicken for purebreeding programs with good breeding practices. Despite this, the government and related stakeholders hold regular Mirah chicken contests to increase the economic value of Mirah chickens and develop their population. In conclusion, Mirah chickens must be conserved because this chicken is related to the socio-culture of Simalungun society. Hence, the pure breeding program at the Mirah chicken farms plays an important role in protecting two original genetic resources of this chicken, i.e., Bankiva and Gallus clades.

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