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DNA Barcode of Red Junglefowl *Gallus gallus* L, 1958 (Aves: Phasianidae) of Sumatra Based on Mitochondrial COI DNA Gene

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Abstract. Genetic data of red junglefowl from southern Sumatra is valuable for conservation, unfortunately the data is not yet available. The purpose of this study was to elucidate genetic character, single nucleotide polymorphism, genetic distance, and phylogeny of red junglefowl based on mtDNA COI gene. Blood samples (20 individuals) of red junglefowl were taken from Bengkulu Province and South Sumatra Province from May to November 2021. Total DNA isolation followed the procedure of the Spin-Column Protocol Kit, Qiagen. DNA replication using the Polymerase Chain Reaction technique with specific primers. The results revealed 716 conserved, 16 variable, 9 parsimony, and 6 singleton sites from the 732 bp nucleotide sequence. Six specific sites (SNPs) as barcodes for Sumatran Junglefowl were found at sequences 51, 273, 327, 721, 729, and 732. The mean genetic distance between species was 7.4%. The red junglefowl of South Sumatra Province and Bengkulu Province are closely related with 98% bootstrapping and separated from another *Gallus* in the same group (ingroup) with 100% bootstrap. Red junglefowl from southern Sumatra has genetic differences from other chickens in the world and these differences can be used as a species barcode and as origin identification the widely traded red junglefowl.

Key words: barcoding; deforestation; genetic conservation; illegal trading; Phasianidae

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INTRODUCTION

The red junglefowl *Gallus gallus* (Phasianidae) is a bird with a reddish-orange dominant body color. Male body size is around 65-78 cm and females of 41-46 cm. Dark brown wings, metallic green tail, and dark underparts. This bird is the ancestor of domesticated chickens with red irises, a brownish beak, and bluish-gray feet. Range across South to South-East Asia in disturbed forests, secondary forests, shrubs, and agricultural land (Eaton et al., 2016). The bird is closely related to the *Gallus varius, G. lavayetii,* and *G. sonneratii* (Wang et al., 2013; Li et al., 2015).

Genetic data of red junglefowl from southern Sumatra (Bengkulu and South Sumatra Provinces) are important to develop *Burgo* chickens into its own family of Indonesian chickens. These data are needed to explain genetic characters, single nucleotide polymorphism (SNP), genetic distance, and phylogeny between each other in the same family. In addition, the data can also be used as a supporting document for the proposed determination of the original *Burgo* chicken breed in Bengkulu and its surrounding areas. However, genetic data and phylogeny of the southern Sumatran red junglefowl as the ancestor of the *Burgo* chicken (Sulandari & Zein, 2009) are not yet available in the world's molecular libraries, either NCBI or the BOLD System.

On the other hand, the existence of red junglefowl in natural habitats (in situ) is highly threatened. The threat comes from the loss of main habitat, namely secondary forest and shrubs (Putranto et al., 2017). The main habitat is converted into plantation land, settlements, roads, and mining. As a result, these birds live with limited habitat resources, both in terms of area and food. Limited habitat and food resources cause population decline. Natalia et al. (2020) found that the density of red junglefowl in Tonusu village, Central Sulawesi was only 0.04 individuals per hectare. Massive hunting by the community has also led to a declined population of red junglefowl in Sumatra's natural habitat. This bird is caught for various purposes, including as a partner of local hens with their offspring (F1) called *Burgo* chickens, consumption, and trade (Setianto et al., 2017). Usually, hunters use fishing nets, rifles (Setianto et al., 2016; Suhadi, 2019), and snares installed in their habitats and playgrounds.

Research that reveals the genetic data of red junglefowl in Indonesia is still limited. Sulandari & Zein (2009) found 26 specific haplotypes of local Indonesian chickens from 72 haplotypes identified in the mitochondrial DNA (mtDNA) D-loop fragment. The quality of genomic DNA in mtDNA COI and Cyt b genes based on a ratio of 260 per 280 from blood samples of red junglefowl from North Sulawesi ranged between 1.807-1.880 and 1.857-1.883, respectively (Kamagi, 2017). Studies using microsatellite genes that have been carried out include Dorji et al. (2012); Riztyan et al. (2014); Phuc & Berres (2018); and Lawal et al. (2018). However, studies with genetic markers of the mtDNA COI gene have not been carried out. For this reason, this study is the first in the world, especially for red junglefowl south of Sumatra. This gene is a coding sequence that can explain species characters, nucleotide polymorphisms, genetic distance, and phylogeny between animal taxa, and evolution (Sulandari et al., 2008; Hata et al., 2021). Genetic characters could be determined based on morphological characters, protein profiles, and molecular traits (Susanti et al., 2017). The mitochondrial COI gene is the most genetic markers widely use for the genetic populations and phylogeography research in the animal kingdom (Hariyantoet al., 2019).

The aim of this study was to describe the genetic character, single nucleotide, genetic distance, and phylogeny between the southern red junglefowl of Sumatra and other species of Phasianidae based on the mitochondrial COI DNA gene. We hope that the sequences produced can be used as a means of species identification and determination of the origin of the traded red junglefowl. In addition, this data can also be used for data to support the determination of the Bengkulu endemic burgo chicken breed. On a global scale, the sequences generated can fill the gaps in the molecular libraries on the NCBI and BOLD System sites.

METHODS

Study area

This study was carried out from May to November 2021. Blood samples (±0.5 ml) from 20 individuals of red junglefowl were taken from the living collections of the community of Bengkulu Province (Central Bengkulu and Seluma Regencies) and South Sumatra Province (North Musi Rawas Regency) via carpal joint vein following the Bengkulu University ethical clearance protocol No. 50/KEH-LPPM/EC/2021. Blood samples were preserved using a vacutainer tube filled with EDTA and stored in a freezer at -20 °C, before a further analysis at the Molecular Biology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Bengkulu University. In addition to the above samples, the analysis also included data of two individuals of *Gallus gallus*, two other species of the genus Gallus, and 9 individuals of 7 other Phasianidae species as the sequences were downloaded from GenBank.

Isolation and purification

Blood samples from the 20 birds were preserved in EDTA vacutainer tube at -20 °C as much as 15-20 µl was transferred into a 1.5 mL Eppendorf tube. The Spin-Column Protocol was used to isolate of genome DNA, applied with *Dneasy® Blood and Tissue Kit*, and its catalogue number 69 504 (50) procured from Qiagen, Germany.

Amplification and sequencing

The COI gene nucleotide in total genome was replicated using the polymerase chain reaction (PCR) procedure to detect any difference therein. In this process, a pair primer designed through Primer 3 (accessed on http://bio-info.ut.ee/primer 3-0.4.0/primer3), while gene alignment was guided by Red Junglefowl *Gallus gallus* gene from GenBank (accession no. KM096864) were used. The primers were named GGCOIF (5'-GCCCATGCTTTCGTCATAAT-3') and GGCO-IR (5'-CTCGGGTGTCTACGTCCATT-3') and produced in 730 bp nucleotides. The amplification was performed in *SimpliAmpTM Thermal Cycler* machine, Applied Biosystems. The reaction mixture consisted of 9.8 µl ddH2O, 5.0 µl Enhancer, 5.0 µl Qs buffer, 1.0 µl dNTP, 1.0 µl forward and reverse primer (20 pmol/µl), 0.2 µl Taq polymerase, and 3 µl DNA template. The PCR temperature sequences applied were 95 °C for pre-denaturation (4 minutes), 94 °C for denaturation (1 minute), 56 °C for annealing (45 seconds), and 72 °C for extension (1 minute). The amplified DNA was migrated to 1.2% agarose gel in 1x TBE (Tris base-Boric acid-EDTA) solution using *Submarine Electrophoresis Hoefer, USA*. This agarose gel electrophoresis refers to Ghaheri et al. (2016). Amplification product with clear band was sent to First Base laboratory (Malaysia) for sequencing.

Table 1. Number of individual Red Junglefowl for analysis and sampling locations

No.	Species	Sample Code	Blood Vol. (ml)	District	Province/Location
1.	*Gallus gallus*	AHB1	0.5	Seluma	Bengkulu
2.	*Gallus gallus*	AHB2	0.5	Seluma	Bengkulu
3.	*Gallus gallus*	AHB3	0.5	Seluma	Bengkulu
4.	*Gallus gallus*	AHB4	0.5	Seluma	Bengkulu
5.	*Gallus gallus*	AHB5	0.5	Central Bengkulu	Bengkulu
6.	*Gallus gallus*	AHB6	0.5	Central Bengkulu	Bengkulu
7.	*Gallus gallus*	AHB7	0.5	Central Bengkulu	Bengkulu
8.	*Gallus gallus*	AHB8	0.5	Central Bengkulu	Bengkulu
9.	*Gallus gallus*	AHB9	0.5	Central Bengkulu	Bengkulu
10.	*Gallus gallus*	AHB10	0.5	Central Bengkulu	Bengkulu
11.	*Gallus gallus*	AHB11	0.5	Central Bengkulu	Bengkulu
12.	*Gallus gallus*	AHB12	0.5	Central Bengkulu	Bengkulu
13.	*Gallus gallus*	AHS1	0.5	North Musi Rawas	South Sumatra
14.	*Gallus gallus*	AHS2	0.5	North Musi Rawas	South Sumatra
15.	*Gallus gallus*	AHS3	0.5	North Musi Rawas	South Sumatra
16.	*Gallus gallus*	AHS4	0.5	North Musi Rawas	South Sumatra
17.	*Gallus gallus*	AHS5	0.5	North Musi Rawas	South Sumatra
18.	*Gallus gallus*	AHS6	0.5	North Musi Rawas	South Sumatra
19.	*Gallus gallus*	AHS7	0.5	North Musi Rawas	South Sumatra
20.	*Gallus gallus*	AHS8	0.5	North Musi Rawas	South Sumatra
21.	*Gallus gallus*	KM096864	-	-	GenBank
22.	*Gallus gallus*	GU261698	-	-	GenBank
23.	*Gallus varius*	NC007238	-	-	GenBank
24.	*Gallus sonneratii*	NC007240	-	-	GenBank
25.	*Pavo muticus*	EU417811	-	-	GenBank
26.	*Pavo cristatus*	NC024533	-	-	GenBank
27.	*Pavo cristatus*	KF444060	-	-	GenBank
28.	*Lophura swinhoii*	NC023779	-	-	GenBank
29.	*Lophura bulweri*	MW574373	-	-	GenBank
30.	*Lophophorus impejanus*	NC040850	-	-	GenBank
31.	*Lophophorus impejanus*	MF975712	-	-	GenBank
32.	*Coturnix japonica*	NC003408	-	-	GenBank
33.	*Coturnix pectorallis*	MW574362	-	-	GenBank

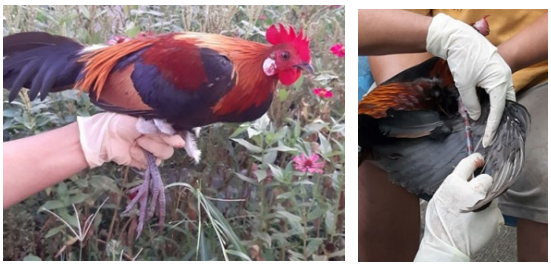

Figure 1. One of the red unglefowl individuals and the process of taking blood samples through the carpal joint vein.

Data analysis

Editing and alignment of nucleotides were performed using *Clustal W* run through *MEGA 10.2.2 software* (Kumar et al., 2018). Nucleotide sequences were checked and trimmed by BIOEDIT software version 7.0.9 (Hall et al., 2011). All sequence samples (20 individual) were successfully aligned into 732 bp before imported to the Barcode of Life Database (BoLD) System in http://www.barcodinglife.org website to explore their similarity. The genetic distances were calculated based on the Kimura 2-parameter (K2P) method (Kumar et al., 2018).). Neighbour-Joining (NJ) models with 1000 bootstrap repetitions was used for reconstruct the phylogenetic tree (Kumar et al. 2018). Two full length COI gene sequences of the Red Junglefowl (*Gallus gallus*, GenBank Accession Number KM096864 and GU261698) and two others species in *Gallus* genus (*Gallus varius*, GenBank Accession Number NC007238

and *Gallus sonneratii* GenBank Accession Number NC007240) downloaded from GenBank were used as ingroup and nine COI gene sequences from different species in Phasianidae were used as outgroup (Table 1).

RESULTS AND DISCUSSION

Product size sequence and sample identification

The product size of the mtDNA CO1 gene sequence was determined through the species-specific primer design of the red junglefowl. The length of the product size was set at 730 bp. All samples with clear and targeted PCR product bands were continued to the sequencing stage. Sequencing results showed that the length of each individual nucleotide sequence (n=20) varied, for forward it was between 699 and 704 bp and reverse 698 and 706 bp. After being evaluated and corrected using primers, 732 bp of nucleotide sequences were found to be suitable for analysis. The length of the nucleotide sequence used for the analysis is included in the range of sequence lengths that are often used in barcoding based on the mtDNA COI gene in animals (Gonçalves et al., 2015; Zein 2018; Jarulis et. al., 2018).

All samples (n=20) analyzed showed the correctness of the species name based on the similarity results in the BOLDSystem. The similarity value of the 20 samples tested ranged from 99.30-99.62% (Table 2). The bird species identified in the database are *Gallus gallus* from China and Canada. The level of similarity of the COI gene sequences of the red junglefowl population from Bengkulu with the GenBank database was higher than the population of South Sumatra. Generally, two taxa are declared as distinct species if they have a sequence difference of 3.0% (Kress et al., 2014; Zein, 2018). However, in some animals the threshold can be smaller (Jarulis et al., 2018; Zein, 2018). In addition to showing that the sample analyzed was *Gallus gallus*, these results also illustrate that the DNA sequence of the COI gene from Bengkulu and South Sumatra Provinces is not yet available in the GenBank database. Therefore, the sequences found can be used as a comparison in identifying red junglefowl in Indonesia.

Nucleotide variation and composition

The nucleotide variation of the COI gene (732 bp) of 20 red junglefowl from southern Sumatra is shown in Table 3. From Table 3 it can be seen that there are 716 (97.81%) conserved sites, 16 (2.18%) variable sites, 9 (1.22%) parsimony sites, and 6 singleton sites (0.83%). The number of sites that

Table 2. Species identification result based on BoLD System database

Species	Sample Code	Species Identified	Similarity (%)	BIN ID	Country
Gallus gallus 1	AHB1	*Gallus gallus*	99.62	AAA3630	China
Gallus gallus 2	AHB2	*Gallus gallus*	99.62	AAA3630	China
Gallus gallus 3	AHB3	*Gallus gallus*	99.62	AAA3630	China
Gallus gallus 4	AHB4	*Gallus gallus*	99.62	AAA3630	China
Gallus gallus 5	AHB5	*Gallus gallus*	99.62	AAA3630	China
Gallus gallus 6	AHB6	*Gallus gallus*	99.62	AAA3630	China
Gallus gallus 7	AHB7	*Gallus gallus*	99.43	AAA3630	China
Gallus gallus 8	AHB8	*Gallus gallus*	99.62	AAA3630	China
Gallus gallus 9	AHB9	*Gallus gallus*	99.62	AAA3630	China
Gallus gallus 10	AHB10	*Gallus gallus*	99.43	AAA3630	China
Gallus gallus 11	AHB11	*Gallus gallus*	99.43	AAA3630	China
Gallus gallus 12	AHB12	*Gallus gallus*	99.43	AAA3630	China
Gallus gallus 13	AHS1	*Gallus gallus*	99.47	AAA3630	Canada
Gallus gallus 14	AHS2	*Gallus gallus*	99.47	AAA3630	Canada
Gallus gallus 15	AHS3	*Gallus gallus*	99.47	AAA3630	Canada
Gallus gallus 16	AHS4	*Gallus gallus*	99.47	AAA3630	Canada
Gallus gallus 17	AHS5	*Gallus gallus*	99.47	AAA3630	Canada
Gallus gallus 18	AHS6	*Gallus gallus*	99.30	AAA3630	Canada
Gallus gallus 19	AHS7	*Gallus gallus*	99.30	AAA3630	Canada
Gallus gallus 20	AHS8	*Gallus gallus*	99.47	AAA3630	Canada

Note: BIN: Barcode Index Number

Table 3. Conservative and variable site and nucleotide composition of the CO1 gene *Gallus gallus* at 732 bp length

Species	Sample Code	Conserved Site	Variable Sites			Nucleotide Composition (%)			
			V	Pi	S	A	T	G	C
Gallus gallus 1	AHB1					25.5	26.1	16.3	32.1
Gallus gallus 2	AHB2					25.5	26.1	16.3	32.1
Gallus gallus 3	AHB3					25.5	26.1	16.3	32.1
Gallus gallus 4	AHB4					25.5	26.1	16.3	32.1
Gallus gallus 5	AHB5					25.5	26.1	16.3	32.1
Gallus gallus 6	AHB6					25.5	26.1	16.3	32.1
Gallus gallus 7	AHB7					25.7	26.1	16.1	32.1
Gallus gallus 8	AHB8					25.5	26.1	16.3	32.1
Gallus gallus 9	AHB9					25.4	26.1	16.4	32.1
Gallus gallus 10	AHB10	716	16	9	6	25.7	26.1	16.1	32.1
Gallus gallus 11	AHB11					25.8	26.1	16.0	32.1
Gallus gallus 12	AHB12					25.7	26.1	16.1	32.1
Gallus gallus 13	AHS1					25.8	26.1	16.1	32.0
Gallus gallus 14	AHS2					25.7	26.1	16.1	32.1
Gallus gallus 15	AHS3					25.7	26.1	16.0	32.2
Gallus gallus 16	AHS4					25.5	26.1	16.2	32.2
Gallus gallus 17	AHS5					25.7	26.1	16.0	32.2
Gallus gallus 18	AHS6					26.0	26.1	15.7	32.2
Gallus gallus 19	AHS7					25.8	26.1	15.9	32.2
Gallus gallus 20	AHS8					25.7	26.1	16.0	32.2
Average						25.64	26.1	16.14	32.13
Total						51.7		48.3	

Note: V=variable, Pi=parsimony informative, S=singleton, A=adenine, T=tymine, G=guanine, C=cytosine.

conserved the COI gene found was similar to the previous studies. In the COI gene sequence (650 bp) of the genus Calidris and Tringa (Aves: Scolopacidae) there were 221 variable sites, 211 of which were parsimoniously informative sites (32.46%) (Huang & Tu, 2016).

The nucleotide composition of each individual from twenty Gallus gallus samples was quite varied (Table 3). The composition of Adenine (A) for all individuals was between 25.4 and 26.0% (mean 25.64%), Thymine (T) 26.1%, Guanine (G) between 15.7 and 16.4%, and Cytosine (C) 32.0-32.2%. Guanine composition was the lowest of the other three nucleotides. The percentage of nucleotide base pairs Adenine and Thymine (AT) was higher than that of guanine and cytosine (GC), 51.7% and 48.3%, respectively. The average nucleotide composition in the COI gene genera Calidris and Tringa were 27.11% T, 31.09% C, 25.57% A and 16.23% G (Huang & Tu, 2016). The base composition of adenine (A), thymine (T), cytosine (C), and guanine (G) was 27.5%, 23.6%, 32.5%, and 16.4%, respectively in Parrot (Psittaciformes). Ashari & Astuti (2017) reported that the base cytosine has the highest percentage (31.7%) and guanine is the lowest (16.6%) in 666 bp COI gene. In addition, the composition and frequency of nucleotides found in this study were similar to those found in mitochondrial genes of other bird groups and in the COI of other animals (Huang & Tu, 2016; Jarulis et al., 2018).

Single nucleotide polymorphism

The results of the alignment of the CO1 gene sequences from 20 individuals of *Gallus gallus* showed the presence of single nucleotide polymorphism (SNP) or specific nucleotide (Table 4). The total nucleotide sites that differed between the red junglefowl and the two other Phasianidae species (*Gallus sonneratii* and *G. varius*) were six sites, namely site 51, 273, 327, 721, 729, and 732. These SNPs could be used as species barcodes to distinguish the red junglefowl from other types of chickens belong to Sumatran Phasianidae family. Barcodes between animal species can be explained by the diversity of nucleotide sequences of the mtDNA COI gene (Kress et al., 2014, Huang & Ruan, 2017). According to Waugh (2007) each species is unique in its mtDNA COI gene sequence and usually only slightly different.

Genetic distance

The genetic distance of the Phasianidae interspecies found in this study is in accordance with several previous studies on barcoding (Tables 5, 6). Table 5 showed that the average genetic distance of interspecies in the *Gallus* genus was

Table 4. Single nucleotide polymorphism of *Gallus gallus* CO1 gene with 732 bp length

Species	Sample Code	Nucleotide site					
		51	**273**	**327**	**721**	**729**	**732**
Gallus sonneratii	NC007240	C	A	C	G	G	C
Gallus varius	NC007238	C	A	C	G	G	C
Gallus gallus 1	AHB1	T	G	T	A	C	G
Gallus gallus 2	AHB2	T	G	T	A	C	G
Gallus gallus 3	AHB3	T	G	T	A	C	G
Gallus gallus 4	AHB4	T	G	T	A	C	G
Gallus gallus 5	AHB5	T	G	T	A	C	G
Gallus gallus 6	AHB6	T	G	T	A	C	G
Gallus gallus 7	AHB7	T	G	T	A	C	G
Gallus gallus 8	AHB8	T	G	T	A	C	G
Gallus gallus 9	AHB9	T	G	T	A	C	G
Gallus gallus 10	AHB10	T	G	T	A	C	G
Gallus gallus 11	AHB11	T	G	T	A	C	G
Gallus gallus 12	AHB12	T	G	T	A	C	G
Gallus gallus 13	AHS1	T	G	T	A	C	G
Gallus gallus 14	AHS2	T	G	T	A	C	G
Gallus gallus 15	AHS3	T	G	T	A	C	G
Gallus gallus 16	AHS4	T	G	T	A	C	G
Gallus gallus 17	AHS5	T	G	T	A	C	G
Gallus gallus 18	AHS6	T	G	T	A	C	G
Gallus gallus 19	AHS7	T	G	T	A	C	G
Gallus gallus 20	AHS8	T	G	T	A	C	G

Note: The number one sequence of Red Junglefowl is equivalent to the number 181 complete sequence of the *Gallus gallus* COI gene (access code KM096864) from GenBank.

0.074 (7.4%). The average genetic distance between Bengkulu and South Sumatra was 0.001 (0.1%) and interpopulation (between Bengkulu and South Sumatra populations) was 0.004 (0.4%). Furthermore, the mean intergenus genetic distance in the family Phasianidae was 0.155 (15.5%). The above intrapopulation, interpopulation, interspecies, and intergenus genetic distances are in line with many previous studies (Waugh 2007; Gonçalves et al, 2015; Huang & Tu, 2016; Susanti et al., 2018; Jarulis et al., 2018). Abinawanto et al. (2022) reported that the genetic distance of intra population of *ayam ketawa* in Bangkalan District ranged from 0.025 to 1.722. For native chickens population in Labuhan Batu District between 0.048-2.736 (Rangkuti et al., 2016) and between native chickens population (*Gallus gallus domesticus*) in the Philippines 0.019-1.367 (Bondoc & Santiago, 2012).

Genetic distances between species in the Charadrius genus were more than 8% (Ashari & Astuti, 2017). Genetic distance between individuals based on ND2 on *Trichoglossus ornatus* birds ranged from 0.001% to 0.008%. Brison et al., (2009) stated that close genetic distance describes the close relationship between the nucleotide sequences of the taxon being compared. Furthermore, if the genetic distance between two taxa is <0.1 then it is said to be closely related (Nei & Kumar, 2000). The value of genetic distance supports grouping, the closeness of each individual in the population, and between these groups and differences in nucleotides (Abinawanto et al., 2022).

Table 5. Average genetic distance between species in Phasianidae based on of CO1 gene with 732 bp length

Genetic Distance	Minimum	Maximum	Average
Intrapopulation in Bengkulu	0.000	0.004	0.001
Intrapopulation in Palembang	0.005	0.014	0.004
Interpopulation	0.000	0.014	0.008
Interspecies *Gallus*	0.064	0.080	0.074
Intrafamily *Phasianidae*	0.143	0.174	0.155

Table 6. Pairwise distance matrix between species in Phasianidae based on of CO1 gene with 732 bp length

Sample code	*Pairwise distance*																														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**
AHB6																																	
AHB8	0.000																																
AHB5	0.000	0.000																															
AHB4	0.000	0.000	0.000																														
AHB1	0.000	0.000	0.000	0.000																													
AHB2	0.000	0.000	0.000	0.000	0.000																												
AHB11	0.003	0.003	0.003	0.003	0.003	0.003																											
AHB7	0.001	0.001	0.001	0.001	0.001	0.001	0.001																										
AHB10	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000																									
AHB12	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.000																								
AHB9	0.001	0.001	0.001	0.001	0.001	0.001	0.004	0.003	0.003	0.003																							
AHB3	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.001																						
AHS1	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.012	0.012	0.012	0.010	0.008																					
AHS2	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.014	0.014	0.014	0.011	0.010	0.001																				
AHS6	0.011	0.011	0.011	0.011	0.011	0.011	0.008	0.010	0.010	0.010	0.012	0.011	0.005	0.007																			
AHS7	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.005	0.005	0.005	0.008	0.010	0.007	0.008	0.004																		
AHS4	0.007	0.007	0.007	0.007	0.007	0.007	0.010	0.008	0.008	0.008	0.005	0.007	0.004	0.005	0.007	0.003																	
AHS3	0.005	0.005	0.005	0.005	0.005	0.005	0.008	0.007	0.007	0.007	0.007	0.008	0.005	0.007	0.005	0.001	0.001																
AHS5	0.005	0.005	0.005	0.005	0.005	0.005	0.008	0.007	0.007	0.007	0.007	0.008	0.005	0.007	0.005	0.001	0.001	0.000															
AHS8	0.005	0.005	0.005	0.005	0.005	0.005	0.008	0.007	0.007	0.007	0.007	0.008	0.005	0.007	0.005	0.001	0.001	0.000	0.000														
KM096864	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.015	0.015	0.015	0.012	0.011	0.011	0.012	0.017	0.015	0.012	0.014	0.014	0.014													
GU261698	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.015	0.015	0.015	0.012	0.011	0.011	0.012	0.017	0.015	0.012	0.014	0.014	0.014	0.003												
NC007238	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.017	0.017	0.017	0.014	0.012	0.015	0.017	0.021	0.019	0.017	0.018	0.018	0.018	0.004	0.007											
NC007240	0.074	0.074	0.074	0.074	0.074	0.074	0.074	0.076	0.076	0.076	0.073	0.071	0.074	0.076	0.080	0.079	0.076	0.077	0.077	0.077	0.065	0.065	0.064										
EU417811	0.137	0.137	0.137	0.137	0.137	0.137	0.137	0.139	0.139	0.139	0.135	0.133	0.137	0.138	0.143	0.142	0.139	0.140	0.140	0.140	0.132	0.132	0.130	0.141									
NC024533	0.142	0.142	0.142	0.142	0.142	0.142	0.142	0.144	0.144	0.144	0.140	0.139	0.142	0.143	0.149	0.147	0.144	0.145	0.145	0.145	0.134	0.137	0.132	0.146	0.017								
KF444060	0.142	0.142	0.142	0.142	0.142	0.142	0.142	0.144	0.144	0.144	0.140	0.139	0.142	0.143	0.149	0.147	0.144	0.145	0.145	0.145	0.134	0.137	0.132	0.146	0.017	0.000							
NC023779	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.152	0.152	0.152	0.148	0.147	0.150	0.152	0.157	0.155	0.152	0.154	0.154	0.154	0.142	0.142	0.142	0.172	0.160	0.156	0.156						
MW574373	0.140	0.140	0.140	0.140	0.140	0.140	0.140	0.141	0.141	0.141	0.138	0.136	0.140	0.141	0.146	0.145	0.141	0.143	0.143	0.143	0.131	0.131	0.131	0.142	0.148	0.151	0.151	0.055					
NC040850	0.161	0.161	0.161	0.161	0.161	0.161	0.161	0.163	0.163	0.163	0.160	0.158	0.161	0.163	0.168	0.167	0.163	0.165	0.165	0.165	0.153	0.153	0.153	0.165	0.148	0.150	0.150	0.161	0.150				
MF975712	0.161	0.161	0.161	0.161	0.161	0.161	0.161	0.163	0.163	0.163	0.160	0.158	0.161	0.163	0.168	0.167	0.163	0.165	0.165	0.165	0.153	0.153	0.153	0.165	0.148	0.150	0.150	0.161	0.150	0.000			
NC003408	0.157	0.157	0.157	0.157	0.157	0.157	0.157	0.159	0.159	0.159	0.155	0.153	0.157	0.158	0.164	0.162	0.158	0.160	0.160	0.160	0.150	0.150	0.148	0.157	0.171	0.172	0.172	0.174	0.153	0.158	0.158		
MW574362	0.148	0.148	0.148	0.148	0.148	0.148	0.148	0.150	0.150	0.150	0.147	0.145	0.148	0.150	0.155	0.153	0.150	0.152	0.152	0.152	0.142	0.138	0.142	0.135	0.165	0.171	0.171	0.173	0.155	0.143	0.143	0.087	

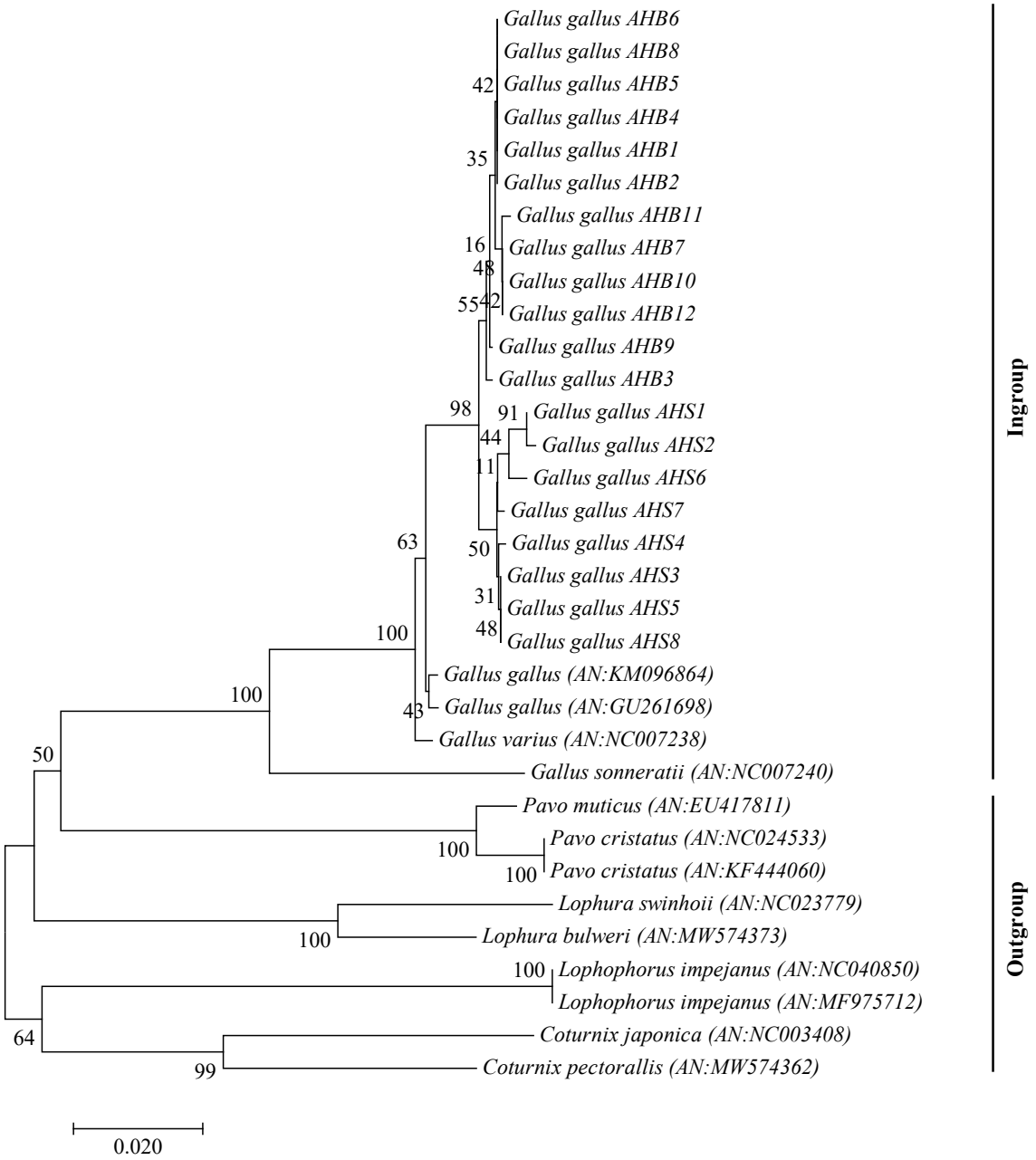

Figure 2. Neighbor Joining phylogenetic tree of 20 reconstructed *Gallus gallus* individuals using a1000 times bootstrap K2P model based on the CO1 mtDNA gene (732 bp).

Relationship

The phylogenetic tree reconstructed using the Kimura-2 parameter Neighbor-Joining tree model with 1000 bootstrap shows a clear separation between Sumatran junglefowl and other chickens (Figure 2). Sumatran junglefowl are in clade subgroups 2 and 3, while the other junglefowl are in subgroup 3. Red junglefowl from Bengkulu province are quite clearly separated from junglefowl from South Sumatra Province. All species of the genus Gallus join in an ingroup cluster. While the outgroup cluster is filled by members of other Phasianidae species. These results indicate that the Bengkulu subpopulation red junglefowl is separate from the South Sumatra subpopulation. A clear separation was also discovered between the red junglefowl species and other species in the family Phasianidae with a bootstrap value of 47-100%. The boostrap value is a benchmark for explaining the level of trustworthiness and stability of the evolutionary process between groups (clades) in the phylogeny tree (Abinawanto et al., 2022). According to Graur

& Li (2010), the hereditary relationship between taxon is described by tree branches and the evolutionary relationship between two nodes by the length of the phylogenetic tree.

Our research has succeeded in revealing genetic information of red junglefowl from Sumatra, especially from southern Sumatra, which was not previously available. The COI gene sequences that we found proved that there were six different nucleotide sites between the Sumatran red junglefowl and two other species of chickens of the genus *Gallus*, and this is the novelty of our study. This red junglefowl COI gene sequence could be used to identify the same species of chicken molecularly. Our sequences data also could be implemented in dealing with the currently high trade in red junglefowl in Sumatra, particularly in tracking the origin of traded red junglefowl.

CONCLUSION

Our study showed that there were 716 conserved sites, 16 variable sites, 9 parsimony sites, and 6 singleton sites from the 732 bp nucleotide sequence. Six specific sites (SNPs) as barcodes for Sumatran Junglefowl were found at sequences 51, 273, 327, 721, 729, and 732. The mean genetic distance between individuals was 0.1%, between populations was 0.8%, between species was 7.4%, and between genera was 15.5%. The red junglefowl of South Sumatra Province and Bengkulu Province are closely related with 98% bootstrapping and separated from another *Gallus* in the same group (ingroup) with 100% bootstrap. The *Gallus-gallus* group is quite far apart from the outgroup species in the Phasianidae family with 47-100% bootstrap. Red junglefowl from southern Sumatra has genetic differences from other chickens in the world and these differences can be used as a species barcode and as origin identification the widely traded red jungle fowl.

In order to achieve the goal of conservation of this species in natural habitats, several studies need to be carried out: genetic study using complete mtDNA and nuclear DNA, a study of the size of the wild population in the Bengkulu and South Sumatra provinces, and a study of their diet, ecology, behavior, and spatial distribution. All data on these topics is still very limited.

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