



Genetic variation of Burgo chicken from Bengkulu, Indonesia, based on the *ND1*-mitochondrial DNA gene

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Abstract: Burgo chicken is one of the domesticated red jungle chickens found in Bengkulu Province, Indonesia. Taxonomically, the position of Burgo chicken as a subspecies, species or breed remains unclear due to the lack of supporting data, highlighting the need for further taxonomic identification. We identified two specific sites, 52 and 375, representing single nucleotide polymorphisms in the *ND1* gene, with a gene sequence length of 450bp. Three haplotypes were detected in Burgo chickens, with haplotype 2 shared between Burgo chicken, *Gallus gallus* (Java) and *G. gallus* bankiva. The average genetic distance in the Burgo chicken population was 0.1%. When compared to other chicken populations, the average distance was 0.12%, while the distance to other *Gallus* spp. was 3.62%. All Burgo chickens formed the same clade in the phylogenetic tree, although two individuals (C2F3ND1 and K4F2ND1) showed slight differences. These two individuals were found in Rejang Lebong and Kepahiang, two nearby locations, indicating the possibility that a meeting occurred. Genetic differences within Burgo chickens from Bengkulu, and with other chickens in Indonesia and various parts of the world, were present but not significant. Our data show that Burgo chickens may exhibit differences from other chickens in Indonesia and globally. However, although the genetic data revealed some divergence in mitochondrial DNA, additional morphological and morphometric analyses are needed to provide supporting evidence.

Keywords: Burgo chickens, conservation, domestication, genetics, taxonomy

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Introduction

The domestication of wild animals is part of the journey of human civilization. One of the most commonly domesticated animals is the chicken. Chickens are bred for egg and meat production. The red partridge is the first chicken that was successfully domesticated in Southeast Asia and Southwest China (Fumihito *et al*, 1994; Väisänen *et al*, (2005); Liu *et al*, 2006; Miao *et al*, 2013). Studies indicate that the domestication process of red partridges in Asia began around 3,000 years ago, leading to the species now known as the domestic chicken (*Gallus gallus domesticus*). Domestication of

the red partridges (*Gallus* spp) in East Asia occurred in the mid-late Holocene. (Miao *et al*, 2013; Larson *et al*, 2014). Domestication has influenced changes in the behaviour, physiology and productivity of chickens; however, some similarities persist between domestic chickens and their ancestors, such as aggressive behaviour during mating and urinary protein excretion, which remain consistent with that of their wild counterparts. (Al-Nasser *et al*, 2007). Meanwhile, local chickens found in Indonesia have continued to develop since this successful domestication process.

This situation has led to Indonesian chickens forming a different genetic clade from other chickens in Asia. Therefore, Indonesia is considered one of the centres of chicken domestication in Asia (Sulandari *et al*, 2007). In

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Indonesia, there are red partridges (*G. gallus bankiva* and *G. gallus spadiceus*) and green jungle fowl (*G. varius*) with a total of 31 strains spread across the regions of Sumatra, Java, Bali and Nusa Tenggara. (Sibley and Monroe, 1990; Nataannjaya, 2000). One of the local chicken breeds found in Indonesia is the Burgo chicken. Burgo chickens are fertile and can produce a high number of offspring, as well as five times more eggs than the red partridges, averaging 32 eggs per period (Sutriyono, 2016). In addition, Burgo chickens have a distinctive crowing sound and beautiful feather colours, which encourage people to raise them as ornamental animals and livestock. The Burgo chicken population is found in all districts of Bengkulu Province, Sumatra Island (Putranto et al, 2017). However, there has been no research into their genetic relationship and characteristics, so it remains unclear whether it is the result of inherited genetics or the impact of environmental factors. As a source of germplasm, Burgo chickens are threatened by various anthropogenic factors, including habitat fragmentation, which causes isolation in these species, further threatening their populations. Moreover, as one of the local chicken clades, the taxonomic position of Burgo chickens remains unknown. Taxonomic determination is generally based on morphological and genetic characteristics. Studies related to the morphology of Burgo chickens have been conducted previously (Rafian et al, 2017; Safitra et al, 2022). Mitochondrial DNA (mtDNA) has been widely used to analyze genetic variation between populations and species due to the high number of DNA copies, making it suitable for analysis with a limited amount of DNA or easily degraded DNA (Ni'mah et al, 2016). One of the mtDNA genes used for species identification is NADH Dehydrogenase subunit 1 (*ND1*) (Amin and Mushlih, 2020). The *ND1* gene is part of complex I, also known as NADH. Ubiquinone oxidoreductase is the first and largest enzyme complex in the mitochondrial respiratory chain, playing a role in oxidizing NADH to release electrons that assist in the translocation of protons to the inner membrane, producing proton gradients (Hirst, 2010).

The genetic diversity of the *Gallus* genus, based on the mitochondrial DNA COI gene, shows a genetic similarity of 98% between red partridges from Bengkulu and South Sumatra (Jarulis et al, 2022). Several previous studies have utilized the *ND1* gene. For instance, Bowles and Mcmanus (1993) revealed inter- and intraspecies variations in *Echinococcus* from 59 isolates; Raharjo et al (2018) detected rat meat contamination in meatballs using the *ND1* gene; and Widayanti et al (2022) successfully identified mutations at three sites within the 972-nucleotide sequence of the *ND1* gene of Indonesian catfish. Therefore, we investigated the potential of the *ND1* gene to determine the level of genetic similarity among Burgo chicken populations, other chickens in Indonesia, and other *Gallus* species. No comparative genetic study of Burgo chickens, particularly based on the mitochondrial DNA *ND1* gene, has ever been conducted. Therefore, this research is essential to provide data on the genetic diversity and variation among Burgo chicken populations and between species of the *Gallus* genus in Indonesia. The findings will support the Bengkulu Provincial Government's efforts to identify and designate the Bengkulu Burgo chicken cluster for submission to the central government, as part of future conservation initiatives aimed at preserving the population's genetic diversity.

Materials and methods

Blood collection

Blood samples were collected from 28 Burgo roosters owned by members of the Bengkulu Burgo chicken hobbyists. There three locations where the Burgo chicken samples were taken are Bengkulu city, Kepahiang, and Rejang Lebong. Blood samples were drawn through the carpal joints and pectoralis veins. Preserved using EDTA tube according to Seutin et al (1991) and stored in a freezer at -20°C, before use. All blood samples were analyzed in the Molecular Biology Laboratory, Department of Biology, Universitas Bengkulu.

DNA extraction and purification

The blood samples (10-20µl) were preserved in EDTA tubes. The DNA was isolated using the Dneasy® Blood and Tissue Kit Cat. No. 69504 (50), following the Spin-Column Protocol Qiagen procedure with modification. In our research, the elution solution used was 50µl with three repetitions. The isolated DNA was observed on 1.2% agarose gel using electrophoresis and stored in a freezer at -20°C, before the amplification process.

Polymerase chain reaction (PCR) DNA

The *ND1* gene of Burgo chickens was replicated using a PCR technique with a DNA template derived from the total DNA product. The *ND1* gene sequence used to design the specific primer in this study was obtained from the complete genome of mitochondrial DNA from *G. gallus* from Kalimantan (GenBank accession number KY039421). *ND1* The primers were BRND1F (5'CCCACCCTAACAAACCTTCTAATC-3') and BRND1R (5'TAGGGTGACTTCGTAT GAGAT TGT-3'), which amplified a 450bp fragment of the 974 bp *ND1* sequence. All reaction mixtures followed the existing protocol Gotaq green. The reaction mixture contained 25µl Gotaq Green, 1.5 µL forward primer, 1.5µl reverse primer, 3µl DNA template, and 19µl nuclease-free water. PCR amplification was performed using a SimpliAmp Thermal Cycler with the following programme: denaturation at 94°C (1 minute), annealing at 55°C (45 seconds) and elongation at 72°C (1 minute) for 30 cycles. Furthermore, the successful amplification samples were sent to PT. Genetika Sains for sequencing.

Data analysis

The BIOEDIT 7.0.9 software (Hall, 1999) was applied to edit the *ND1* gene sequence and visualize the electrograms and nucleotide base sequences. The nucleotide sequence (forward and reverse) products were aligned using *Clustal W* of the *MEGA 11.0* programme (Tamura et al, 2013). Each individual's gene sequence was compared with the *ND1* reference to determine the similarity level of the samples. The genetic distance between individuals was calculated using the 2-parameter Kimura (K2P) method (Kimura, 1980). The phylogeny tree was constructed using the neighbour-joining (NJ) method with 1,000 replications (Tamura et al, 2013). Additional *ND1 G. gallus* gene sequences found in GenBank were downloaded and included in the phylogenetic tree reconstruction analysis (see Table 1). Genetic diversity parameters, namely haplotype (Hd) and nucleotide (π) diversity were calculated using *DNASp v6.12.03* software

(Rozas *et al*, 2017). The haplotype analysis was presented in a sequence location distribution map/operational taxonomic unit (OTU) and haplotype network images to depict the latest connectivity and genetic distribution between populations using model median-joining by *Network v10.2.0.0* software (Bandelt *et al*, 1999).

Results

Single nucleotide polymorphism

The nucleotide sequence of the *ND1* gene observed in

450bp between Burgo chicken species from Bengkulu had two nucleotide polymorphisms (SNP) that differed among individuals at positions 52 and 375 (Table 1). Site 52 showed a transversion substitution in Burgo chicken individuals from Rejang Lebong Regency and Kepahiang Regency, namely from cytosine (C) to adenine (A), while a transition substitution at site 375 was found among Burgo chicken individuals from Central Bengkulu, namely from the nucleotide base adenine (A) to guanine (G).

Table 1. SNP between individuals of Burgo chickens from Bengkulu based on the *ND1* gene (450bp). Sample code indicates accession numbers of sequences sourced from GenBank. Dots (.) indicate identical nucleotide to the reference sequence for *ND1* (KY039420.1). A, adenine; C, cytosine; G, guanine.

No.	Sample code	Location/Source	Local name	Site number		Haplotype group
				52	375	
1	KY039420.1	GenBank	red junglefowl	C	A	Hap 2
2	KY039418.1	GenBank	red junglefowl	.	.	Hap 2
3	KY039422.1	GenBank	red junglefowl	.	.	Hap 4
4	KY039421.1	GenBank	Red junglefowl	.	.	Hap 5
5	AP003323.1	GenBank	Bankiva	.	.	Hap 2
6	NC007238.1	GenBank	Green junglefowl	.	.	Hap 6
7	NC007240.1	GenBank	Grey junglefowl	.	.	Hap 7
8	NC007239.1	GenBank	Ceylon junglefowl	.	.	Hap 8
9	BR1	Central Bengkulu	Burgo	.	G	Hap 1
10	BR2	Central Bengkulu	Burgo	.	.	Hap 2
11	BR3	Central Bengkulu	Burgo	.	.	Hap 2
12	BR4	Central Bengkulu	Burgo	.	.	Hap 2
13	BR5	Central Bengkulu	Burgo	.	G	Hap 1
14	BR6	Central Bengkulu	Burgo	.	.	Hap 2
15	BR7	Central Bengkulu	Burgo	.	.	Hap 2
16	BR8	Central Bengkulu	Burgo	.	.	Hap 2
17	BR9	Central Bengkulu	Burgo	.	.	Hap 2
18	BR10	Central Bengkulu	Burgo	.	.	Hap 2
19	BR11	Central Bengkulu	Burgo	.	.	Hap 2
20	BR12	Central Bengkulu	Burgo	.	.	Hap 2
21	BR13	Central Bengkulu	Burgo	.	.	Hap 2
22	BR14	Central Bengkulu	Burgo	.	G	Hap 1
23	BR15	Central Bengkulu	Burgo	.	.	Hap 2
24	C1F2	Rejang Lebong	Burgo	.	.	Hap 2
25	C2F3	Rejang Lebong	Burgo	A	.	Hap 3
26	C4F2	Rejang Lebong	Burgo	.	.	Hap 2
27	C5F2	Rejang Lebong	Burgo	.	.	Hap 2
28	K1F1	Kepahiang	Burgo	.	.	Hap 2
29	K2F1	Kepahiang	Burgo	.	.	Hap 2
30	K3F2	Kepahiang	Burgo	.	.	Hap 2
31	K4F2	Kepahiang	Burgo	A	.	Hap 3
32	K5F2	Kepahiang	Burgo	.	.	Hap 2
33	K10F2	Kepahiang	Burgo	.	.	Hap 2
34	K11F2	Kepahiang	Burgo	.	.	Hap 2
35	K12F2	Kepahiang	Burgo	.	.	Hap 2
36	K13F3	Kepahiang	Burgo	.	.	Hap 2

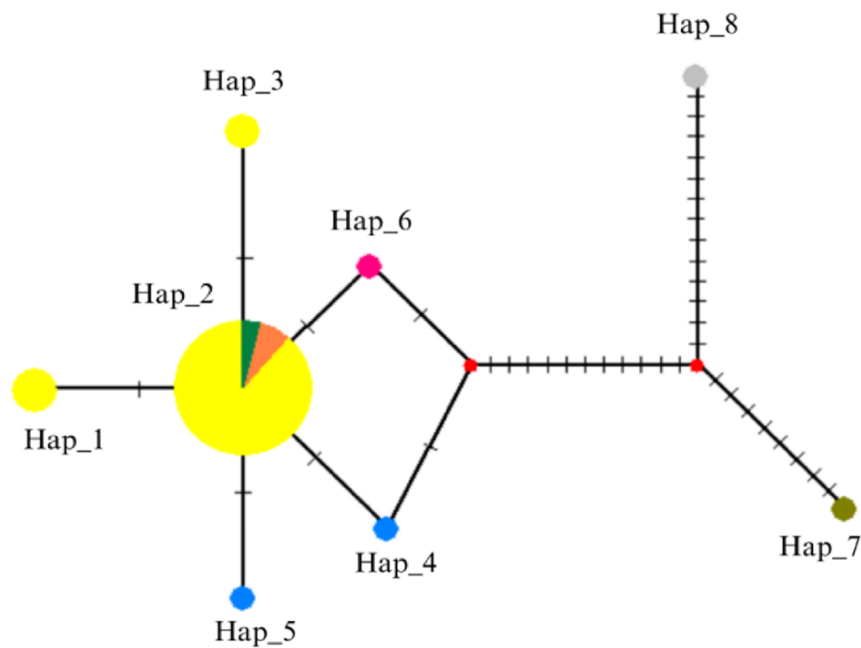


Figure 1. The haplotype network of the *Gallus* spp. population, based on the *ND1* gene alignment. Yellow, Burgo chicken (this study); blue, *G. gallus* from Kalimantan (KY039422.1; KY039421.1); green, *G. gallus bankiva* (AP003323.1); orange, *G. gallus* from Java (KY039420.1; KY039418.1); red, *G. varius* (NC007238.1); grey, *G. sonneratii* (NC007240.1); army green *G. lafeyetii* (NC007239.1).

Haplotype network

In this study, network reconstruction was performed using median-joining (Bandelt et al, 1999). Twenty-eight samples of Burgo chickens were complemented with additional genetic data of eight samples of the *Gallus* genus from Genbank, including *G. gallus* from Kendu (KY039420.1), *G. gallus* from Garut (KY039418.1), *G. gallus* from Nunukan (KY039422.1), *G. gallus* from Tarakan (KY039421.1), *G. gallus bankiva* (AP003323.1), *G. varius* (NC007238.1), *G. sonneratii* (NC007240.1), and *G. lafeyetii* (NC007239.1). We succeeded in identifying eight haplotypes with a sequence length of 450bp. In Burgo chicken samples, three haplotypes were found: hap 1, hap 2 and hap 3 (Figure 1). Each haplotype is separated by a single nucleotide base, represented by a small horizontal line connecting the haplotypes.

Genetic distance

Genetic distances were analyzed using pairwise distances with the *MEGA 11* software (Table 2). In general, genetic distance is divided into three groups, namely genetic distance between individuals (intraspecific), genetic distance between *G. gallus* species, and genetic distance between species of the *Gallus* genus (interspecific). In this study, a slight change was observed in the interspecific genetic distance compared to all Burgo chicken samples, incorporating genetic data from red partridges and subspecies from GenBank. Meanwhile, the outgroup comparison involved all red partridges and their offspring with all other partridges. The intraspecific genetic distance among three districts in Bengkulu Province, based on the *ND1* gene, showed the lowest value of 0%, while the highest distance was 0.4%.

Table 2. Intra- and interspecific genetic distance in Burgo chickens based on the *ND1* gene (450bp)

Genetic Distance	Maximum	Minimum	Average
Intrapopulation of Burgo chicken	0.4%	0.0%	0.1%
Burgo chicken versus other <i>Gallus gallus</i>	0.4%	0.0%	0.12%
Interspecies of <i>Gallus</i> spp.	5.9%	0.2%	3.62%

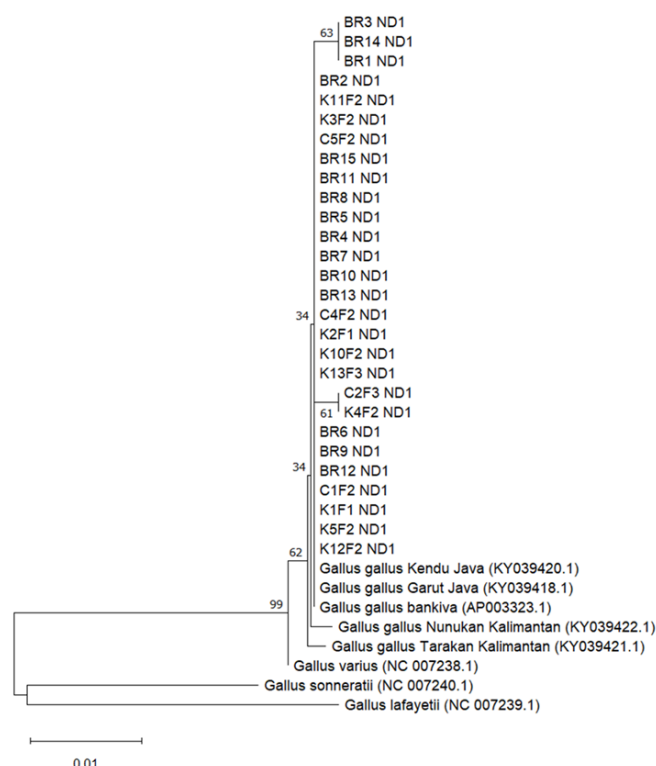
Phylogeny

Phylogenetic trees were reconstructed using the *ND1* gene to determine the taxonomic position of Burgo chickens in comparison with the available reference data. The phylogeny of the Burgo chicken sample was grouped into one clade with red partridge from Java, Kalimantan, and the subspecies of *G. gallus bankiva* (Figure 2). However, five samples formed a small group, namely BR3, BR14 and BR1 from Central Bengkulu, and C2F3 and K4F2 from Rejang Lebong and Kepahiang. This is due to the discovery of mutations in the sequence that caused a slight change; however, the shape of the phylogenetic tree remains stable.

Discussion

Human intervention drives the continuous domestication of chickens. Domestication has led to the development of many chicken breeds worldwide. Indonesia has 31 local chicken breeds that have adapted over tens to hundreds of years. Each local chicken breed has characteristics influenced by its specific region (Nataamijaya, 2010). These characteristics are intrinsically linked to their genetic foundation, such as SNPs. SNPs are often used to interpret variations and identify species or individuals (Torres, 2016). In our study, there were two differentiation sites, namely sites 52 and 375. At site 52, a change was present in nucleotide bases from C to A (C2F3 and K4F2), while a change from A to G was found at site 375 (BR1, BR5, and BR14). These changes are caused by mutations. According to Warmadewi *et al* (2020),

Figure 2. Phylogenetic tree construction with neighbour-joining (NJ) modelling of 28 Burgo chickens from Bengkulu using a K2P model and 1,000-time bootstrap, based on the *ND1* gene (450bp).



mutations can enhance adaptability by eliminating original traits. Sometimes, the treatment such as maintaining high stocking density and implementing accelerated growth diets of domestic chickens has negative impacts such as health problems, brittle bones, and even sudden death (Hirsch, 2003; Meseret, 2016). However, it is not yet known for certain whether the changes that occur in Burgo chickens have a positive or negative impact on their ability to adapt, so more in-depth research is needed regarding the morphometry and morphology of Burgo chickens. The C2F3 and K4F2 samples were Burgo chickens obtained from Kepahiang and Rejang Lebong Districts, while the BR1, BR5 and BR14 samples were Burgo chickens from Central Bengkulu District. The landscapes in each location differ: Kepahiang and Rejang Lebong are highland areas, whereas Central Bengkulu is a lowland area, leading to different adaptation processes.

Based on the SNP data, Burgo chickens are grouped into three haplotypes according to their sequence similarity, namely hap 1, hap 2 and hap 3. The 450bp alignment of the *ND1* gene yielded eight haplotypes of the entire sequence (Figure 1). Similar genetic data is present from several species, including Burgo chicken, *G. gallus* from Java, and *G. gallus bankiva*. This is interesting because several Burgo chickens share the same genetic components as *G. gallus* (Java) and *G. gallus bankiva*; however, the results of the haplotype analysis may be influenced by the number of samples and population. Research by Wang *et al* (2020) using 863 native and domestic chicken genomes showed that crossbreeding occurred among red partridge subspecies. Therefore, it is possible that all three originated from the same ancestor. These data are strengthened by previous studies that revealed the origin of red partridge as the ancestors of local chickens worldwide. Sulandari *et al* (2008) found 69 haplotypes in the genetic characterization of local Indonesian chickens and local chickens outside Indonesia using D-loop, besides discovering the Indonesian chicken genes in other countries. Although our data only used three breed populations in Bengkulu Province, they revealed a direct relationship between Burgo chickens, red partridge, and their descendants, as indicated by haplotype 2.

Genetic distance is one of the tools used for species identification, alongside morphological and morphometric data. Lately, bird research has been relying on genetic data to facilitate the identification process. Each species has a threshold value for genetic distance; if the genetic distance is equal to or greater than 3%, species separation occurs (Fouquet *et al*, 2007). Based on genetic distance identification, the distance between Burgo chickens and red partridges (intersubspecific) is 0.1–0.4%. Meanwhile, the distance among Burgo chickens (intraspecific) ranges from 0 to 0.4%, indicating a close relationship at the species level. This suggests that they likely originate from a closely related or similar population. Therefore, Burgo chicken can be identified as a new breed of red partridge. However, further study on morphometry as well as sound identification is required to ensure this theory. Similarly, Utama *et al*, (2023) obtained the genetic distance between Burgo chickens and red partridges as 0–0.8% using the COI gene. In addition, Zein and Sulandari (2008) reported that the genetic distance between native chicken populations in Lombok, using the D-loop, ranged from 0.1–1.7%. The distance between red partridges from Bengkulu and South Sumatra based on the COI gene has also been confirmed to range from 0–1.4%

(Jarulis et al, 2022). The genetic distance between red partridges and domesticated individuals, as measured by the D-loop and COI gene, exhibits a divergence range of 0–1.7%, which is higher than the divergence observed in the *ND1* gene. Therefore, the *ND1* gene is more conserved.

The results of the phylogenetic tree reconstruction of 28 Burgo chickens from Bengkulu using the NJ model and 1,000 bootstraps are presented in Figure 2. NJ is one of the phylogenetic analysis methods based on the difference in the evolution rate of each branch. The components in NJ analysis are the operational taxonomic units and evolutionary distance. Based on the phylogenetic tree, all Burgo chickens form a large clade, joined by *G. gallus bankiva* and *G. gallus* from Kalimantan and Java. This suggests that, genetically, Burgo chicken still have a direct relationship as descendants of the red partridge. However, there are two small groups among the individual Burgo chickens, due to nucleotide base differences at sites 52 and 375. Several factors can cause differences in nucleotide bases, including geographical and environmental factors, as well as the duration of isolation, all of which can trigger mutations. In general, the genes used for identification are the COI gene and the non-coding region (D-loop). Many studies have focused on these two genes (Zein and Sulandari, 2012; Bilgin et al, 2016). However, several previous studies have stated that the *ND1* gene can also be used for identification because it contains conserved regions (Bowles and Mcmanus, 1993; Raharjo et al, 2018; Widayanti et al, 2022). Therefore, the use of the *ND1* gene in species identification can be applied as an alternative to the COI gene with more stable traits in the region. However, this method is not yet accurate in determining the taxonomic position or discovering species history because our study only used 450bp ($\pm 50\%$) of the total length of the *ND1* gene (974bp).

Conclusion

The *ND1* gene sequence of mitochondrial DNA from the original Burgo chicken in Bengkulu has been successfully obtained. SNPs were identified at two sites of the *ND1* gene, with a sequence length of 450bp. The average genetic distance within the Burgo chicken population was 0.1%, while the distance between Burgo chicken to other chicken populations was 0.12%. All Burgo chickens formed the same clade in the phylogenetic tree, though two individuals (C2F3 and K4F2) showed slight differences, forming small groups based on variations in nucleotide bases. Genetic differences among Burgo chickens from Bengkulu, other chicken species in Indonesia, and several locations worldwide are present but non-significant. Our data show that Burgo chickens may be genetically distinct from other chickens found in Indonesia and globally. However, further research on the morphology and morphometrics of Burgo chickens is needed to confirm these findings.

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Author contributions

Jarulis and Ahmat Fakhri Utama contributed to the study's conception and design. Data collection was done by Jarulis. Data analysis and writing of the first manuscript draft were performed by Jarulis and Ahmat Fakhri Utama. All authors commented on the various versions of the manuscript, and read and approved the final manuscript.

Ethics statement

All experiments were approved by the local ethics committee of the University of Bengkulu, Indonesia. Animal procedures were conducted in accordance with the ethical code guidelines No. 15/KER-LPPM/EC/2023.

Conflict of interest statement

The authors declare no conflicts of interest.

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