

# Genetic diversity analysis of Thai indigenous chickens based on complete sequences of mitochondrial DNA D-loop region

Piyanat Teinlek<sup>1,2</sup>, Kannika Siripattaraprat<sup>1,2,3</sup>, and Chanin Tirawattanawanich<sup>1,2,4,\*</sup>

\* **Corresponding Author:** Chanin Tirawattanawanich  
**Tel:** +66-2-579-7538, **Fax:** +66-2-579-7538,  
**E-mail:** [fvetcnt@ku.ac.th](mailto:fvetcnt@ku.ac.th)

<sup>1</sup> Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand

<sup>2</sup> Center of Excellence on Agricultural Biotechnology (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand

<sup>3</sup> Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand

<sup>4</sup> Department of Physiology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand

**ORCID**

Piyanat Teinlek

<https://orcid.org/0000-0002-1198-4741>

Kannika Siripattaraprat

<https://orcid.org/0000-0002-5847-2356>

Chanin Tirawattanawanich

<https://orcid.org/0000-0002-1570-4380>

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**Objective:** Complete mtDNA D-loop sequences of four Thai indigenous chicken varieties, including Pra-dhu-hang-dam (PD), Leung-hang-khao (LK), Chee (CH), and Dang (DA) were explored for genetic diversity and relationships with their potential ancestor and possible associates to address chicken domestication in Thailand.

**Methods:** A total of 220 complete mtDNA D-loop sequences of the four Thai indigenous chicken varieties were obtained by Sanger direct sequencing of polymerase chain reaction amplicons of 1,231 to 1,232 base pair in size. A neighbor-joining dendrogram was constructed with reference complete mtDNA D-loop sequences of Red Junglefowl (RJF) and those different chicken breeds available on National Center for Biotechnology Information database. Genetic diversity indices and neutrality test by Tajima's D test were performed. Genetic differences both within and among populations were estimated using analysis of molecular variance (AMOVA). Pairwise fixation index ( $F_{ST}$ ) was conducted to evaluate genetic relationships between these varieties.

**Results:** Twenty-three identified haplotypes were classified in six haplogroups (A-E and H) with the majority clustered in haplogroup A and B. Each variety was in multiple haplogroups with haplogroups A, B, D, and E being shared by all studied varieties. The averaged haplotype and nucleotide diversities were, respectively 0.8607 and 0.00579 with non-significant Tajima's D values being observed in all populations. Haplogroup distribution was closely related to that of RJF particularly *Gallus gallus gallus* (*G. g. gallus*) and *G. g. spadiceus*. As denoted by AMOVA, the mean diversity was mostly due to within-population variation (90.53%) while between-population variation (9.47%) accounted for much less. By pairwise  $F_{ST}$ , LK was most closely related to DA ( $F_{ST} = 0.00879$ ) while DA was farthest from CH ( $F_{ST} = 0.24882$ ).

**Conclusion:** All 4 Thai indigenous chickens are in close relationship with their potential ancestor, the RJF. A contribution of shared, multiple maternal lineages was in the nature of these varieties, which have been domesticated under neutral selection.

**Keywords:** Thai Indigenous Chicken; Mitochondrial DNA D-loop; Genetic Diversity; Domestication

## INTRODUCTION

It is widely believed that domestic chickens are descendants of the red junglefowl (RJF) as firstly indicated by Darwin [1] in the comparisons of morphology and production among Gallus species. Supportive molecular genetic evidence was later reported with a suggestion of monophyletic origin theory in which *Gallus gallus gallus* (*G. g. gallus*), those endemic to Southeast Asia, potentially serves as the sole ancestor of domestic chicken [2,3]. Such information has some limitations as being extracted from small sample sizes of partial mtDNA D-loop sequences of 400 base pairs (bp), even though supportive evidences on the Southeast Asian origination of domestic chickens has been published [4]. Complete sequences of mtDNA D-loop region of Japanese indigenous chickens were later investigated for more

clarification of phylogenetic relationships, maternal origin and route of introduction into Japan in which genetic derivation of Chinese and Korean chickens from Southeast Asia were defined [5]. On the other hand, some researchers suggested the possibility of multiple maternal origins and multiple domestications of domestic chickens from different regions, including India, Southwest China and Southeast Asia [6-10]. Besides *G. g. gallus*, genetic relationships between Indian domestic chickens and *G. g. spadiceus* and *G. g. murghi* have also been reported [9].

Regarding mtDNA D-loop information of Southeast Asian chickens, Indonesian indigenous chickens have been reported to scatter in five clusters, being associated to reference sequences from India, China and Indonesia [11]. Laotian indigenous chickens have also been found dispersed in five clades, having the majority in clades A and B of which locations of origin are believed to be Southeast Asian continent and China [12]. Vietnamese local chickens appear to be more diverse with 8 clusters which related to reference sequences of Indian, China and Southeast Asian origins [13]. These suggested that Southeast Asian indigenous chickens would have multiple maternal origins.

Molecular technology has the efficiency and accuracy in the genetic study among breeds of animals. The evolutionary rate of the mtDNA is about five to ten times faster than the nuclear genome due to the fact that mutation accumulates slowly in nuclear genes [14]. In addition, unlike the mtDNA with the presence of only maternal encoding genetic sequences, the nuclear genes represent the integrative genetic information derived from both parental sides in every generation, which could hide the history of individuals due to recombination. Tracking the history of particular nuclear DNA segment is therefore difficult. The complete sequence of mtDNA D-loop region has been successfully used in genetic study especially to determine phylogenetic relationships, including genetic distance and genetic variability within and among populations [5,7,15]. We therefore explored the complete mtDNA D-loop sequences of Thai indigenous chickens, including Pradhu-hang-dam (PD), Lueng-hang-khao (LK), Chee (CH), and Dang (DA) for genetic diversity and their relationships with other reference sequences of RfJ and domestic chickens. We anticipated gaining significant evidences to better understand chicken domestication in Thailand, which is proposed to be the origin of chicken domestication [2].

## MATERIALS AND METHODS

### Animals and sample collection

The animal use protocol for this study was approved by Kasetsart University Institutional Animal Care and Use Committee. Four Thai indigenous chicken varieties, PD (n = 80), LK (n = 76), CH (n = 34), and DA (n = 30) from reference genetic

stocks of the Department of Livestock and Development of Thailand were studied for their phylogenetic relationships based on complete mtDNA D-loop sequence. These populations have been maintained in a way to ensure that they are good representative genetic resources of each variety. The sequenced birds are parent stocks with no background family relationships among each individual. Blood samples were collected from brachial vein, using heparinized syringes. The samples were transferred on ice and kept frozen at  $-40^{\circ}\text{C}$  for later polymerase chain reaction (PCR) analysis.

### mtDNA D-loop amplification and sequencing

Complete mtDNA D-loop region was specifically amplified by PCR using a primer pair of AV1F2: 5'-AGGACTACGGC TTGAAAAGC-3' and r2-3m: 5'-TGCTTAAGGTTA ATTAC TGCTG-3' [16]. The PCR reaction was performed in a 50  $\mu\text{L}$  reaction volume containing 25  $\mu\text{L}$  of 2 $\times$  Phusion Blood Direct PCR Master Mix (Thermo Scientific, Waltham, MA, USA), 1 pmol/ $\mu\text{L}$  of each primer, 1  $\mu\text{L}$  of the whole blood sample and 22  $\mu\text{L}$  of  $\text{dH}_2\text{O}$ . The PCR was conducted in a thermal cycler (ARKTIK Thermal Cycler, Thermo Scientific, USA) with the conditions of 5 min initial denaturation at  $98^{\circ}\text{C}$ , followed by 35 cycles, each consisting of 1 s denaturation at  $98^{\circ}\text{C}$ , 5 s annealing at  $62^{\circ}\text{C}$  and 1 min extension at  $72^{\circ}\text{C}$  cycle, and then final extension step at  $72^{\circ}\text{C}$  for 5 min.

Each of the amplified product was mixed with SYBR Green (Thermo Scientific, USA) and 6 $\times$  loading dye before subjecting to electrophoresis using 1% agarose gel in 0.5 $\times$  Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer. The amplified products were visualized under ultraviolet light and purified using GeneJET PCR Purification Kit (Thermo Scientific, USA) before sending for direct sequencing on both strands at 1st BASE sequencing service (Singapore).

### Data analysis

All sequences of 220 Thai indigenous chickens were viewed and edited. Multiple alignments and nucleotide sequence variations were conducted by ClustalW and BioEdit Sequence Alignment Editor, and identical sequences were considered as the same haplotype using DnaSP version 5.10 [17]. mtDNA D-loop diversity indices, including the number of haplotype (H), haplotype diversity (Hd), and nucleotide ( $\pi$ ) diversity and Tajima's D test were estimated using DnaSP version 5.10 [17] and Arlequin version 3.5 [18]. To evaluate the sequence variation among and within the population, analysis of molecular variance (AMOVA) and pairwise fixation index ( $F_{ST}$ ) were computed using Arlequin version 3.5 [18].

Molecular phylogenetic analysis of complete D-loop sequences of Thai indigenous chickens and the nucleotide DNA databank from GenBank were conducted with molecular evolution genetic analysis version 6.0 [19]. An unrooted neighbor-joining (NJ) tree was constructed using Kimura 2-parameter

model. Bootstrap values of the phylogenetic tree were estimated with 1,000 repetitions. Classifying haplotype into 13 haplogroups based on the mitochondrial genome study by Miao et al [7] with reference mtDNA sequences from the National Center for Biotechnology Information database as listed in Table 1 was performed. A sequence of Ceylon junglefowl (accession no. NC\_007239) was chosen as outgroup.

## RESULTS

### mtDNA D-loop sequence variability and population diversity

The complete mtDNA D-loop sequences of the studied samples were found to be 1,231 to 1,232 bp in length with a total number of 38 variable sites being identified (Table 2). All variable sites were in transition excepting position 391 that was in transversion and position 859 that was single nucleotide insertion/deletion. A total of 23 haplotypes were identified.

The diversity indices calculated for all experimental individuals are presented in Table 3. A total number of haplotypes of all studied populations was 23, of which 16 (69.57%) were singletons and only 7 (30.43%) were shared between the Thai indigenous chickens. The highest number of haplotypes was found in LK representative population while the lowest number detected was in CH (13 and 4 haplotypes, respectively). The Hd of the Thai indigenous chickens was found ranging

from 0.6399 to 0.8320 with the highest diversity being estimated in PD population and the lowest diversity in CH population. The averaged haplotype diversity of all population was approximately 0.8607. The nucleotide diversity ( $\pi$ ) of the Thai indigenous chickens was between 0.00492 to 0.00563 that the highest value was observed in LK population and the lowest was in DA population. The average nucleotide diversity of the 220 mtDNA D-loop sequences of Thai indigenous chicken populations was estimated to be 0.00579. For all four varieties of Thai indigenous chicken, Tajima's D statistics for neutrality test was not significant ( $p > 0.05$ ).

In Table 4, the genetic variation within and among population being quantified by AMOVA based on Kimura-2 parameter distances is presented. Within population variation was found accounted for 90.53% of the genetic variation while differences among population generated a rather small contribution (9.47%) to total genetic variation of the studied of Thai indigenous chicken populations. Differences among populations were highly significant ( $p < 0.01$ ).

### Genetic distance among Thai indigenous chicken populations

Estimated values of pairwise population differentiation between the four Thai indigenous chickens according to  $F_{ST}$  genetic distances were expressed in Table 5. Among the Thai indigenous chicken varieties, LK population was most closely

**Table 1.** Haplotype names and accession numbers of chicken complete mtDNA D-loop sequences used in this study

Haplotype name	GenBank accession	Breeds/Varieties	Collection sites
RJFspa_A	GU261695	Red junglefowl ( <i>G. g. spadiceus</i> )	China
RJFspa_B	GU261704	Red junglefowl ( <i>G. g. spadiceus</i> )	Myanmar
RJFspa_B	NC_007235	Red junglefowl ( <i>G. g. spadiceus</i> )	Laos
RJFgal_C2	AB007725	Red junglefowl ( <i>G. g. gallus</i> )	Unknown
RJFspa_C3	GU261716	Red junglefowl ( <i>G. g. spadiceus</i> )	Myanmar
RJFmur_C3	GU261707	Red junglefowl ( <i>G. g. murghi</i> )	India
RJFgal_D1	NC_007236	Red junglefowl ( <i>G. g. gallus</i> )	Philippine
RJFban_D1	NC_007237	Red junglefowl ( <i>G. g. bankiva</i> )	Indonesia
RJFmur_E3	GU261708	Red junglefowl ( <i>G. g. murghi</i> )	India
RJFspa_F	GU261702	Red junglefowl ( <i>G. g. spadiceus</i> )	China
RJFspa_F	GU261703	Red junglefowl ( <i>G. g. spadiceus</i> )	Myanmar
RJFspa_G	GU261690	Red junglefowl ( <i>G. g. spadiceus</i> )	China
RJFspa_W	GU261706	Red junglefowl ( <i>G. g. spadiceus</i> )	China
RJFspa_X	GU261692	Red junglefowl ( <i>G. g. spadiceus</i> )	China
RJFspa_Y	GU261693	Red junglefowl ( <i>G. g. spadiceus</i> )	China
RJFjab_Z	GU261674	Red junglefowl ( <i>G. g. jabouillei</i> )	China
ChiNC_C1	GU261701	Domestic chicken	China
ChiNC_D2	GU261683	Domestic chicken	China
IndNC_D3	GU261697	Domestic chicken	India
LaoNC_E1	AP003319	Domestic chicken	Laos
IndNC_E2	HQ857209	Domestic chicken	India
ChiNC_H	GU261715	Domestic chicken	China
JapNC	AB268543	Domestic chicken	Japan
IndNC_I	GU261698	Domestic chicken	India

**Table 2.** Sequence variation among mtDNA D-loop sequences of four varieties of Thai indigenous chicken

Haplotype	Polymorphic nucleotide sites																																								
	167	198	199	210	212	217	219	220	222	225	242	243	246	256	261	281	296	306	310	315	330	342	344	355	363	367	391	396	446	686	792	844	859	966	1075	1178	1214	1215			
A01	C	C	T	C	G	T	C	T	A	T	G	T	C	T	C	A	C	T	C	C	C	A	A	T	C	T	C	T	C	G	G	T	*	G	A	T	C	G			
A02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A03	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
A04	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B01	T	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	A	.	*	.	.	G	.	.	A		
B02	T	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	A	.	*	.	.	.	.	.	A		
B03	T	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	A	.	*	.	.	.	.	.	A		
B04	T	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	A	.	*	.	.	.	.	.	A		
B05	T	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	A	.	*	.	.	.	.	.	A		
B06	T	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	A	.	*	.	.	.	.	.	A		
B07	T	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	A	.	*	.	.	.	.	.	A		
B08	T	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	A	.	*	.	.	.	.	.	A		
B09	T	.	.	A	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	A	.	*	.	.	.	.	.	A		
C01	T	.	.	.	.	.	.	.	.	C	A	C	.	C	.	G	.	.	T	.	.	G	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.		
D01	T	.	.	.	.	.	.	.	.	C	.	C	.	C	T	G	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
D02	T	.	.	.	.	.	.	.	.	C	.	C	.	C	T	G	.	C	T	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
D03	T	.	.	.	.	.	.	.	.	C	.	C	.	C	T	G	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
D04	T	.	.	.	.	.	.	.	.	C	.	C	.	C	T	G	.	C	T	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
E01	T	.	.	.	.	C	.	.	.	C	.	C	.	C	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	
E02	T	.	.	.	.	C	.	.	G	C	.	C	.	C	T	.	.	.	T	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	
E03	T	.	.	.	.	C	.	.	.	C	.	C	.	C	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	
E04	T	.	.	.	.	C	.	.	.	C	.	C	.	C	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	A	T	.	.	.	.	.	.	.	.	.	
H01	T	T	C	.	.	.	.	.	.	C	.	C	.	C	T	.	.	.	C	T	.	.	.	.	.	.	.	.	.	T	T	.	.	.	.	.	.	.	.		

The asterisk marks (\*) mean nucleotide deletions and dots (.) refer to the same nucleotide with the first sequence.

**Table 3.** Diversity indices in four varieties of Thai indigenous chickens based on complete mtDNA D-loop sequences

Item	n	H	Hd	π	Tajima's D (p-value)
Pra-dhu-hang-dam	80	11	0.8320	0.00545	1.31973 (0.909)
Leun-hang-khao	76	13	0.8179	0.00563	0.76207 (0.818)
Chee	34	4	0.6399	0.00503	1.37028 (0.931)
Dang	30	8	0.7816	0.00492	-0.00305 (0.546)
Total	220	23	0.8607	0.00579	0.40273 (0.738)

n, number of sequence; H, number of haplotype; Hd, haplotype diversity; π, nucleotide diversity.

**Table 4.** Analysis of molecular variance (AMOVA) of 220 complete mtDNA D-loop sequences of Thai indigenous chickens

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	F <sub>ST</sub>	p-value
Among population	3	63.715	0.34629 Va	9.47	-	-
Within population	216	715.230	3.31125 Vb	90.53	-	-
Total	219	778.945	3.65754	-	0.09468	0.00

d.f., degree of freedom; F<sub>ST</sub>, fixation index.

Va, among-population variance; Vb, between-individual within-population variance.

related to DA population (F<sub>ST</sub> = 0.00879) while further apart from PD and CH populations (F<sub>ST</sub> = 0.07584 and 0.17277). DA was least related to CH population (F<sub>ST</sub> = 0.24882).

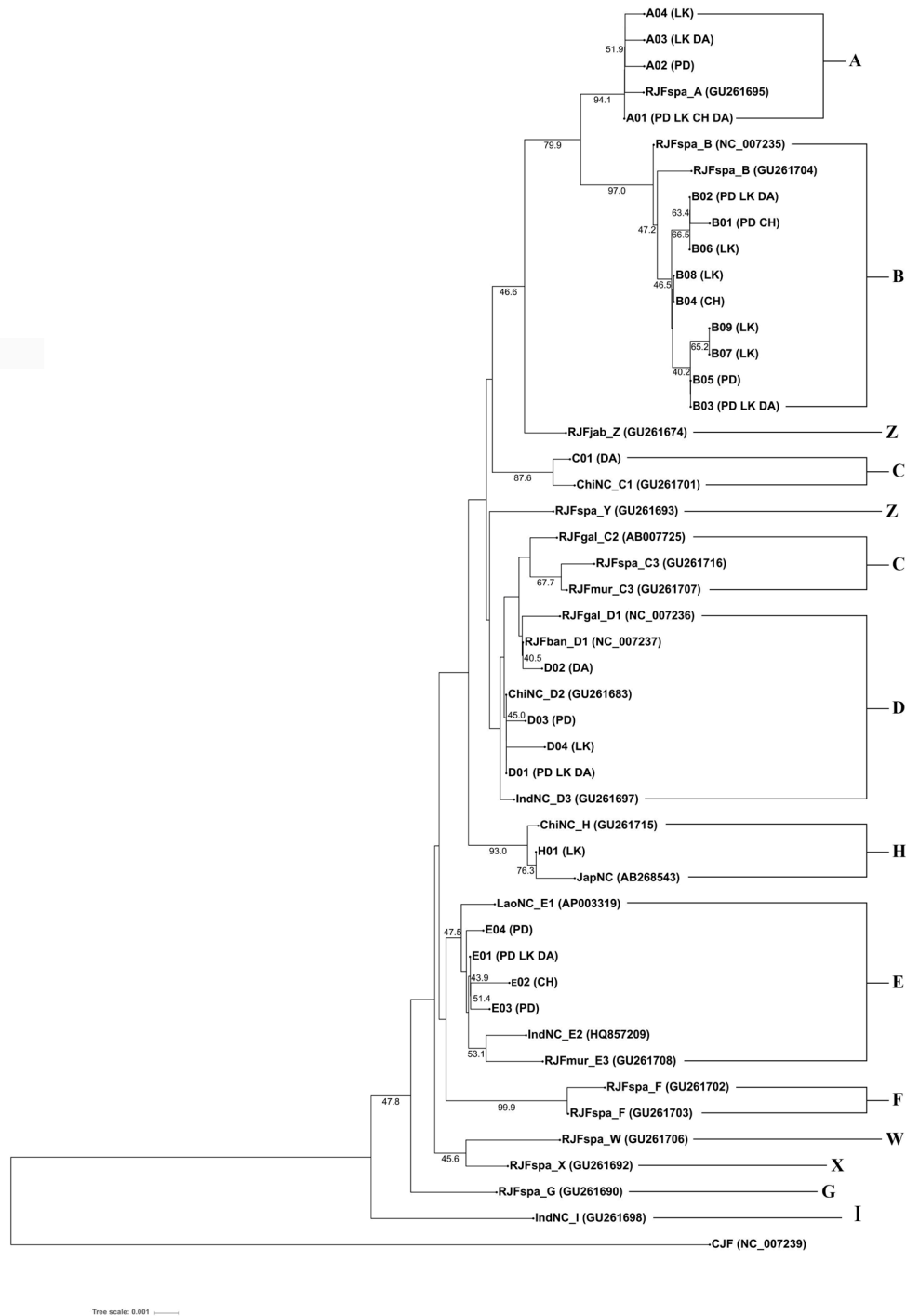
**Table 5.** Pairwise F<sub>ST</sub> between four varieties of Thai indigenous chickens based on mtDNA D-loop sequence

Item	PD	LK	CH	DA
PD	0.00000	-	-	-
LK	0.07584	0.00000	-	-
CH	0.02276	0.17277	0.00000	-
DA	0.12233	0.00879	0.24882	0.00000

F<sub>ST</sub>, fixation index; PD, Pra-dhu-hang-dam; LK, Leung-hang-khao; CH, Chee; DA, Dang.

**Phylogenetic analysis and haplotype distribution**

The phylogenetic analysis (Figure 1) revealed a classification of 23 haplotypes of the Thai indigenous chickens in 6 haplogroups (A-E and H) of the 13-haplogroup setting (haplogroup A-I and W-Z) according to suggestion based on a whole mitochondrial genome study [7]. Haplotype distribution (Table 6) shows that out of these six haplogroups, there were four, including A, B, D, and E, being shared among different varieties, while haplogroups C and H were each represented by only single individual of DA and LK, respectively. Haplogroups A and B were the first and second major haplogroups representing the studied populations at frequencies of 35.45% and 42.27%, respectively. The frequency of haplogroup D was 10.45%, which was comparable to that of haplogroup E, 10.91%.



**Figure 1.** Evolutionary relationships of taxa. The evolutionary history of the 4 Thai indigenous chicken varieties, Pra-dhu-hang-dam (PD), Leung-hang-khao (LK), Chee (CH), and Dang (DA) was inferred using the Neighbor-Joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are displayed next to the branches. Bootstrap values lower than 40% are not shown. Evolutionary analyses were conducted in MEGA6. Data were grouped into 13 haplogroups (A-I and W-Z) as suggested by Miao et al [7].

The majority of the PD and CH representative populations were clustered in haplogroup B, and that of LK and DA were in haplogroup A with frequencies of 53.75% and 67.65%, and 42.11% and 56.67%, respectively. The second major haplogroup of the PD and CH was haplogroup A being accounted re-

spectively for 28.75% and 17.65% while that of LK and DA was haplogroup B for 27.63% and 20%.

Most of PD individuals were in haplotype B01 (31.25%) then followed by haplotypes A01, B02, and A02 which represented 18.75%, 15.0%, and 10% of this variety, respectively.



**Table 6.** Distribution of mtDNA D-loop haplotypes in four varieties of Thai indigenous chickens

Clade	Haplotype	PD	LK	CH	DA	Total
A	A01	15	25	6	13	59
	A02	8	-	-	-	8
	A03	-	2	-	4	6
	A04	-	5	-	-	5
B	B01	25	-	19	-	44
	B02	12	15	-	2	29
	B03	4	2	-	4	10
	B04	-	-	4	-	4
	B05	2	-	-	-	2
	B06	-	1	-	-	1
	B07	-	1	-	-	1
	B08	-	1	-	-	1
	B09	-	1	-	-	1
C	C01	-	-	-	1	1
D	D01	7	9	-	3	19
	D02	-	-	-	2	2
	D03	1	-	-	-	1
	D04	-	1	-	-	1
E	E01	3	12	-	1	16
	E02	-	-	5	-	5
	E03	2	-	-	-	2
	E04	1	-	-	-	1
H	H01	-	1	-	-	1
Total		80	76	34	30	220

PD, Pra-dhu-hang-dam; LK, Leung-hang-khao; CH, Chee; DA, Dang.

Other haplotypes expressed in the PD group, including B03, B05, D01, D03, E01, E03, and E06 each accounted for less than 10%. The haplotypes of LK individuals with frequencies higher than 10% were A01 (32.89%), B02 (19.74%), E01 (15.79%), and D01 (11.84%) while A03 and A04 were represented by less than 10% of this population. The remaining haplotypes including B03, B06, B08, B09, D04, and H01 were each represented by only a single individual. CH individuals were distributed in four haplotypes of which more than half of the samples were in haplotype B02 (55.88%). A01, E02, and B04 haplotypes were accounted for 17.65%, 14.71%, and 11.76% of the CH representative population, respectively. Most of DA individuals were in haplotype A01 (43.33%), and then followed by an equal frequency of 13.33% in haplotype A03 and B03 and 10% in D01. The rest were dispersed in four haplotypes (B02, C01, D02, and E01) with frequencies of less than 10% each.

## DISCUSSION

Chicken domestication has been practiced worldwide for consumption, companionship and entertainment. Human migration introduces a long-distance gene flow and enables genetic material exchanges among chickens of different ori-

gins [6,13]. Exploration of background genetic relationships among chickens in the region might provide traits of human-chicken, human-human and chicken-chicken interactions going on since the prehistoric era and could lead to the identification of an ancestral area as a center of chicken domestication [20,21]. This study presents molecular genetic evidences in supporting the illustration of matrilineal characters and genetic diversity of Thai indigenous chickens as well as their genetic relationships with RJF and other potential associated indigenous chickens.

As revealed by the NJ tree (Figure 1), the 4 Thai indigenous chicken varieties were each distributed in 3 to 5 haplogroups, indicating the existence of a contribution of multiple maternal lineages in all varieties. Each haplogroup was also found representing more than 1 variety, which could suggest that they are derived from common maternal ancestors. All 5 haplogroups are as well represented by RJF, enforcing the role of the RJF as the ancestor of domestic chickens including the Thai indigenous varieties.

The maternal lineage sharing has been reported among different indigenous chicken breeds of various geographical locations [4,8,22]. Identical sequences expressed as the same haplotype of some individuals of different breed/varieties are presented in this study that is in line with other reports [6]. Domestic chickens are therefore suggested to be in close genetic relationships regardless of distinctive phenotypes and physiological/reproductive performance.

Hybridization among chicken breeds with the same or different locality of origin, besides coincident mutations could also be the explanations to the findings above mentioned. Fighting chickens, originally inhabited in Southeast Asia including Thailand, have been commonly crossbred with the expectation of superior performance through hybrid vigor. A trace of this domestication effect could be present in the background genetic relationships among chicken populations. Although, we collected the samples from standard genetic stocks of the Department of Livestock Development of Thailand to ensure their purebred status, breed separation of these indigenous chickens might not have been long enough to express a breed-specific haplogroup in phylogenetic relationship analysis of D-loop mtDNA (Figure 1), which is similar to negative findings in other reports [6].

Non-significant Tajima's D being observed in all studied populations of Thai indigenous chickens (Table 3) signified that they were consistent with a population at mutation-drift equilibrium [23], thereby suggesting neutral selection is involved. The indigenous chickens have been generally raised as free-range backyard flocks in which random mating is permitted without farmer's interest in programmed breeding. The tropical to subtropical environment is also suitable for these chickens to survive and reproduce. Thus, impacts of selection pressures either natural or man-made are less likely applied

on these populations, which supports the neutrality found. AMOVA results (Table 4) suggested that the main contribution to the genetic variance was due to variation within populations. Pairwise genetic relationship as determined by  $F_{ST}$  (Table 5) indicated the highest intimacy between DA and LK (0.00879), while CH was closely genetically related with PD (0.02276). Significant genetic variation (0.24882) between CH and DA was the highest compared to others. It should be noted that DA and CH are less popular among Thai farmers compared to the other two varieties, which are distributed in all parts of the country. DA has been mostly raised in the south while CH has been concentrated in the northeast of the country. This separated geographical distribution could lead to a limited gene flow thereby explaining the least genetic relationships found between DA and CH.

Thai indigenous chickens appeared to be dominated by 2 maternal lineages defined in haplogroup A and B, those having distribution in South and Southwest China and surrounding areas and in Southeast Asia [6,7,12]. About 10% of the Thai indigenous chickens were found in haplogroup D and E indicating that this haplogroup also contributed considerably to Thai indigenous chickens. Haplogroup D has been composed of RJF and gamecocks from Indonesia, India, Japan and various indigenous chickens from Africa, South Asia, Southeast Asia and East Asia of which the origin has been presumed to be in Southwest China and/or surrounding region (Vietnam, Myanmar, Thailand, and India) [6,7]. Haplogroup E is mainly distributed in Eurasian and South Asian domestic chickens [6,7]. The maternal lineages associated with this haplogroup could have originated from the Indian subcontinent and possibly spread to Southeast Asia [6].

Haplogroup C and H were each represented by only one individual of Thai indigenous chicken in this study. Haplogroup C is mainly distributed in East, Southeast and South Asia while haplogroup H has originated in Southwest China and Japan [7]. The haplogroup H is a rare clade with only a few members of gamecocks cited, including Chinese Chigulu and Japanese Shamo breeds [5,7] to which Thai indigenous chickens are added according to this study. Gamecocks of Southeast Asia have been documented as the origin of Okinawa Shamo gamecocks of Japan [24], which were speculated to play a key role as founder of Japanese domestic chickens [25]. On the contrary, Oka et al [5] suggested that this haplogroup which was referred as haplogroup F is isolated to only Okinawa Shamo and has no relationships with other Japanese domestic chickens. Both Southeast Asia and China have been suggested as centers for chicken domestication [2,5], it is therefore interesting to further explore the origin and development of this haplogroup as well as to decipher the relationships between Thais as Southeast Asian chickens and Chinese chickens. Nevertheless, haplogroup H is not documented as being in any of the junglefowl. This leaves the ancestor and domesti-

cation origin of this clade inconclusive, thereby requiring further scrutiny.

In summary, this study suggested that Thai indigenous chickens including DA, CH, PD, and LK are likely to be descended from a common ancestor, the RJF, with multiple matrilineal contributions. The populations were under genetic equilibrium and their genetic variations were mainly under the control of within-population variation. D-loop nucleotide polymorphism appeared to lack breed specific information in this regard.

## CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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