BIODIVERSITAS
Volume 24, Number 4, April 2023
Pages: 1962-1970

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240404

Genetic diversity among five native Thai chickens and Khiew-Phalee chickens in lower-northern Thailand using mitochondrial DNA barcodes

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Manuscript received: 29 January 2023. Revision accepted: 2 April 2023.

Abstract. *Siriwadee P, Wirot L, Thanapol P, Wirawan N. 2023. Genetic diversity among five native Thai chickens and Khiew-Phalee chickens in lower-northern Thailand using mitochondrial DNA barcodes. Biodiversitas 24: 1962-1970.* Native chickens are an important biological resource in Thailand, with each breed having distinctive features. Khiew-Phalee chickens are a valuable investment in cultural wisdom and an invaluable Thai resource worthy of conservation. It is the royal rooster of Phraya Phichai Dab Hak. Due to a crisis concerning a sharp decline in the size of the Khiew-Phalee native chicken population, the identification of potential biomarkers is important for the determination of different chicken breeds for future planning. This study aimed to investigate the molecular identification of different native Thai chicken breeds based on mitochondrial DNA (mtDNA) barcodes, especially Khiew-Phalee chickens, which is difficult using the morphological examination. Partial sequences of 648 bp mtDNA control region fragments were determined for 53 specimens of six breeds of native Thai chickens (Khiew-Phalee, Thao Thong, Leung Hang Khao, Chee, Pra Dhu Hang Dam and Kai Jae). Genetic diversity was accessed based on the number of polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity, and average number of differences. A phylogenetic tree was constructed based on maximum likelihood. The results showed that eighteen haplotypes were identified from 9 polymorphic sites with polymorphism between nucleotides 1074, 1107, 1109, 1177, 1213, 1214, 1268, 1276 and 1305. The haplotype diversity, nucleotide diversity, and the average number of differences for all chickens were 0.917, 0.00504 and 2.69086, respectively. All Khiew-Phalee chickens showed 7 haplotypes with 0.897 haplotype diversity, 0.00696 nucleotide diversity, and an average number of differences at 3.1795 clustered with other breeds in the phylogenetic tree. The findings indicated that the DNA barcoding gene is an effective method to identify the Khiew-Phalee and five other native Thai chicken breeds. It is a better choice for using molecular biology DNA barcoding to identify Thai native chicken breeds to plan for further conservation and development by the Department of Livestock Development of Thailand.

Keywords: Genetic variation, Khiew-Phalee chicken, mitochondrial DNA barcodes, native chicken**,** Thailand

INTRODUCTION

Thai native chickens comprise one of Thailand's most important biological resources. They are valued in terms of rich cultural heritage and biodiversity and are the intellectual property of local Thais. These chickens are continually changing based on natural evolution. As a result, there are many varieties of native chickens, each of which is characterized by unique qualities including outward appearance, resistance to diseases and insects, and the ability to live and propagate under care provided by rural farmers, especially small ones (Likittrakulwong et al. 2019). They are thus apt for conservation and worthy of use in sustainable development. The "Khiew-Phalee Chicken" is one breed of ancient Thai chicken (Phromnoi et al. 2022). It is one of the cultural and intellectual assets in Uttaradit Province in the field of animal agriculture since the Khiew-Phalee chicken is the personal chicken of Father Phraya Phichai Dap Hak. It was also certified by the Department of Livestock Development as local Thai livestock in 2013 as a "local species representing the identity of the province" (Biodiversity Research and Development Section 2014). Thus, the Khiew-Phalee Chicken is a valuable Thai resource and worthy of conservation. Their value can be raised as a protected species or as contest chickens for beauty and enjoyment. They can be raised for recreational competitions (fights). They can be promoted as agricultural products as well to tangibly create jobs to generate income for the people of Uttaradit Province. Nowadays, animal selection uses genetic markers to help detect gene patterns in conjunction with genetic evaluation. To increase efficiency and advancement in animal breeding, this process can be applied to selection and breeding. In Thailand, the hybrid red jungle fowl with the native chicken breeds (Thao Thong, Leung Hang Khao, Chee, Pra Dhu Hang Dam and Kai Jae) has been deliberated as meat types and native chicken breed which is very close to the origin of chicken (Suwannapoom et al. 2018; Likittrakulwong et al. 2019).

The classification of organisms uses morphological and anatomical features to reveal similarities or differences as well as identify species that have been around for a long

time. However, this process might not be able to pinpoint the species due to several limitations. Nowadays, scientists have applied the concept of barcode labeling to science by applying DNA, which is a unique determiner of an organism's characteristics. It is used as a marker to identify organisms or DNA barcodes to increase speed and accuracy in the identification process of species and organisms. It can also increase the efficiency of natural evolution and biodiversity research, including the conservation of natural resources (Hebert et al. 2003). The barcode for each species must be unique to that species, easy to use, and clear. The first gene used as a basis for use of a DNA barcode marker in animals is nucleotides since nucleotide sequences are more distinct between species than in the same species. It is a DNA feature with a conservation area that can allow a universal primer to capture and increase the amount of DNA which is approximately 500-800 base pairs, meaning this gene can be used as a DNA barcode marker for species identification and classification of organisms such as birds and moths (Hebert et al. 2004; Hajibabaei et al. 2006). It has been reported that the mitochondrial DNA (mtDNA) gene contains a hyper-variable displacement loop (D-loop) and cytochrome b (cyt b) gene (Cui et al. 2017; Jin et al. 2022). Tang et al. (2017) reported results that used DNA barcode techniques to identify the genetic diversity of Chinese chicken breeds by using mtDNA D-loop. The mitochondrial cytochrome oxidase subunit I (COI) gene is the genetic marker widely used for genetic populations and phylogeography research in the animal kingdom (Hariyanto et al. 2019; Jaluris et al. 2022).

Phromnoi et al. (2022) found the genetic variability of Khiew-Phalee chickens investigated by using mitochondrial cyt b gene sequences. However, it is not yet an effective approach for examining DNA barcodes for Khiew-Phalee chicken breeds. Consequently, it is important to carry out a study of the genetic diversity of other parts of the DNA barcode gene (bar2 as COI gene) (Cui et al. 2017; Innak et al. 2019) and which gene mitochondrion is different in each chicken breed, and the evolutionary relationship in Uttaradit's Khiew-Phalee chickens, as well as the origin of different breeds of chickens to be used as a guideline for the conservation and enhancement of chicken breeds as well as to determine genetic markers to aid in future chicken breeding programs.

MATERIALS AND METHODS

Study area

The study was conducted in the provinces of Uttaradit and Phitsanulok, located in lower-northern Thailand, which is the origin of the Khiew-Phalee chicken, between January and March 2022 (Figure 1).

Ethics statement

Experimental procedures were approved and conducted following the guidelines of the Animal Ethics Committee, Phibunsongkhram Rajabhat University (Approval reference number: PSRU-(AG)-2021-007).

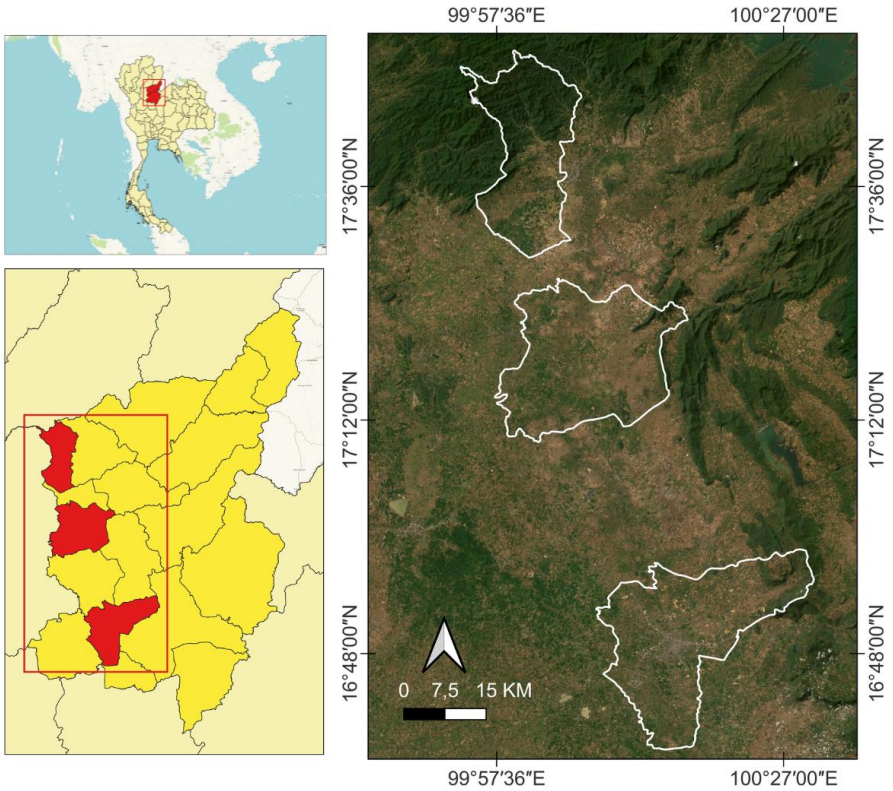

Figure 1. Location of research sites in Laplae and Phichai District, Uttaradit Province and Muang Phitsanulok District, Phitsanulok Province, Thailand

Blood samples collection and DNA isolation

The native chickens used for this study were reared in the semi-free-range condition of the rearing system. A total of 53 native chickens in six breeds were included: Khiew-Phalee (KP, n=13) (Figures 2A and 2B), Thao Thong (GG, n=7) (Figures 2C and 2D), Leung Hang Khao (WTY, n=11) (Figures 2E and 2F), Chee (CH, n=4) (Figures 2G and 2H), Pra Dhu Hang Dam (PD, n=4) (Figures 2I and 2J) and Kai Jae (KT, n=14) (Figures 2K and 2L). Samples were randomly selected by purposive sampling from farmers who raised native Thai chickens on farms and had been raising them continuously for more than 5 years in Uttaradit and Phitsanulok provinces. EDTA blood samples were collected by purposive sampling from the wing vein. Twenty microliters of the collected blood were extracted as total DNA using DNeasy®Blood & Tissue Kit (Qiagen). DNA extraction was performed according to the manufacturing manual, and the elution step used 100 microliters of sterile nuclease-free water. Eluted DNA samples were stored at -20°C until used.

Amplification of a partial mtDNA fragment and nucleotide sequencing

A 648 bp of partial mtDNA control region fragments (DNA barcode gene) by polymerase chain reaction (PCR) used specific primers proposed from previous reports. The forward primer (bar2 F 5′- TCA AGT GAA GCC TGG ACT AC-3′) and reverse primer (bar2 R 5′- TGC GGA TAC TTG CAT GTA TAT- 3′) (Cui et al. 2017). An amplicon of each sample was amplified by Phusion DNA polymerase which had a proofreading activity and analyzed a nucleotide sequence by NGS sequencing based on the MiSeq Illumina sequencing platform at the A T C G Co; Ltd. Bangkok, Thailand. Some samples which gave an unclear DNA sequence, including double-base peak chromatogram and low fragment frequency, were repeated for nucleotide sequencing using the Sanger sequencing method.

The PCR was conducted in a thermal cycler (ARKTIK Thermal Cycler, Thermo Scientific, USA) under the conditions of 5 min initial denaturation at 94°C, followed by 35 cycles, each consisting of 30 s of denaturation at 94°C, 30 s of annealing at 50°C, and 1 min extension at 68°C cycle, and then a final extension step at 68°C for 5 min. The amplified DNA target was analyzed in 2 percent agarose gel electrophoresis to confirm the length of the amplified fragment. Each amplicon was purified using a QIAquick PCR Purification Kit (Qiagen) before being sent for direct sequencing on both strands at the A T C G Co; Ltd. Bangkok, Thailand.

Sequencing analysis and phylogenetic construction

Multiple alignments were performed using the ClastalW program from MEGA version X software among nucleotide sequences based on the DNA barcode gene of the Khiew-Phalee (KP) breed and other breeds obtained from the GenBank database. The samples from each farm were categorized into a haplotype (H) and estimated haplotype diversity (Hd), nucleotide diversity and Tajimas's D test using DnaSP version 6. The genetic distance was calculated by Kimura's 2-parameter method. The neighbor-joining (Nj) method and unweighted maximum parsimony (MP) tree were used to generate the phylogenetic tree with 1000 bootstrap replicas using MEGA version 11 software (Tamura et al. 2021).

RESULTS AND DISCUSSION

Gel electrophoresis of DNA and PCR products

The DNA from the blood of the six chicken breeds was amplified by PCR and verified by gel electrophoresis. The molecular weight of the PCR product from each breed of chicken was about 650 bp proving that DNA was successfully and could be sequenced and alignments.

Mitochondrial DNA sequence analysis

Based on the PCR amplification of 648 bp mtDNA control region fragments, the DNA sequence was performed on 53 specimens of native Thai chickens, and the nucleotide sequence was deposited in the database GenBank under accession numbers OP722518-OP722569, OQ200473 and OQ200474.

When comparing the sequences, this study had 9 different sequence sites (Table 1). Based on the sequence variations, the chickens were divided into 18 haplotypes, most of which fell in haplotype 10, followed by haplotypes 15, 6 and 16, respectively (Table 2). Eighteen haplotypes were identified from 9 polymorphic sites with polymorphism between nucleotides 1074, 1107, 1109, 1177, 1213, 1214, 1268, 1276 and 1305, which had the transition mutation of A<->G or T<->C, as shown in Table 1.

PCR products in 53 samples from six breeds of chickens including Khiew-Phalee, Leung Hang Khao, Pra Dhu Hang Dam, Thao Thong, Chee and Kai Jae compared with 5 samples of NCBI database (CH-1MK163565.1, CH-2MH366570.1, CH-4MH366558.1, CH5-MH366552.1, CH-3EF362663.1) were clustered into 18 haplotypes (Table 2).

Phylogenetic tree of Khiew-Phalee and five other breeds of chicken

Haplotype 1 included all Khiew-Phalee chickens KP1, KP38, KP41 and KP43. Haplotype 2 consisted of 1 sample of Chee chicken (CH26). Haplotype 3 consisted of 2 samples of Khiew-Phalee chicken (KP120 and KP122). Haplotype 4 consisted of 1 sample of Khiew-Phalee chicken (KP121). Haplotype 5 consisted of 1 sample of Khiew-Phalee chicken (KP123). Haplotype 6 consisted of 4 breeds including Khiew-Phalee chicken (KP128 and KP129), Leung Hang Khao (WTY79), Pra Dhu Hang Dam (PD100) and Kai Jae (KT50). Haplotype 7 consisted of 2 breeds comprising Khiew-Phalee chicken (KP132) and Kai Jae (KT54). Haplotype 8 consisted of 2samples of Khiew-Phalee chicken (KP6 and KP134). Haplotype 9 consisted of 2 breeds including Leung Hang Khao (WTY32) and Pra Dhu Hang Dam (PD98).

Figure 2. Different adult Thai native chicken breeds. A. Khiew-Phalee chicken (KP) male and B. female, C. Thao Thong chicken (GG) male and D. female, E. Leung Hang Khao chicken (WTY) male and F. female, G. Chee chicken (CH) male and H. female, I. Pra Dhu Hang Dam chicken (PD) male and J. female and K. Kai Jae chicken (KT) male and L. female

Table 1. Polymorphic sites among haplotypes

Haplotype	Numerical position of nucleotide sequence*									N
	1074	1107	1109	1177	1213	1214	1268	1276	1305	
1	g	g	g	t	c	a	g	g	g	4
2	.	a	.	.	.	.	a	.	a	1
3	a	a	a	.	t	g	a	a	a	2
4	a	a	a	.	.	g	a	a	a	1
5	.	a	a	.	.	.	a	a	a	1
6	a	a	.	.	.	g	a	.	a	5
7	a	a	a	.	.	g	a	.	a	2
8	a	a	.	.	.	.	a	.	a	2
9	a	.	.	c	.	g	.	.	.	2
10	a	.	.	.	.	g	.	.	.	11
11	a	a	a	.	.	.	a	a	a	1
12	a	a	a	.	.	g	a	.	.	1
13	a	a	.	.	.	.	.	.	.	1
14	a	a	.	.	.	g	.	.	.	1
15	a	.	.	.	.	.	.	.	.	8
16	a	a	.	.	.	g	a	.	.	5
17	a	.	.	.	.	g	a	.	.	2
18	a	a	.	.	.	.	a	.	.	4

Note: *The numerical position of each polymorphic site is indicated above each position

Table 2. Samples of each breed in haplotypes based on DNA barcode genes

Haplotypes	Breed					
	KP	WTY	PD	GG	CH	KT
1	KP1 KP38 KP41 KP43	-	-	-	-	-
2	-	-	-	-	CH26	-
3	KP122 KP120	-	-	-	-	-
4	KP121	-	-	-	-	-
5	KP123	-	-	-	-	-
6	KP128 KP129	WTY79	PDD100	-	-	KT50
7	KP132	-	-	-	-	KT54
8	KP6 KP134	-	-	-	-	-
9	-	WTY31	PD98	-	-	-
10	-	WTY32 WTY86 WTY94	-	GG73 GG74 GG75 GG88	-	KT49 KT52 KT58
11	-	WTY83(2)	-	-	-	-
12	-	WTY84	-	-	-	-
13	-	WTY87	-	-	-	-
14	-	WTY93	-	-	-	-
15	-	WTY81 WTY83	PD97 PD99	GG8	CH16	KT24 KT25
16	-	-	-	GG7	-	KT51 KT53 KT56 KT57
17	-	-	-	GG11	CH117	-
18	-	-	-	-	CH113	KT46 KT47 KT48
18	13	11	4	7	4	14

Note: KP: Khiew-Phalee, WTY: Leung Hang Khao, PD: Pra Dhu Hang Dam, GG: Thao Thong, CH: Chee and KT: Kai Jae

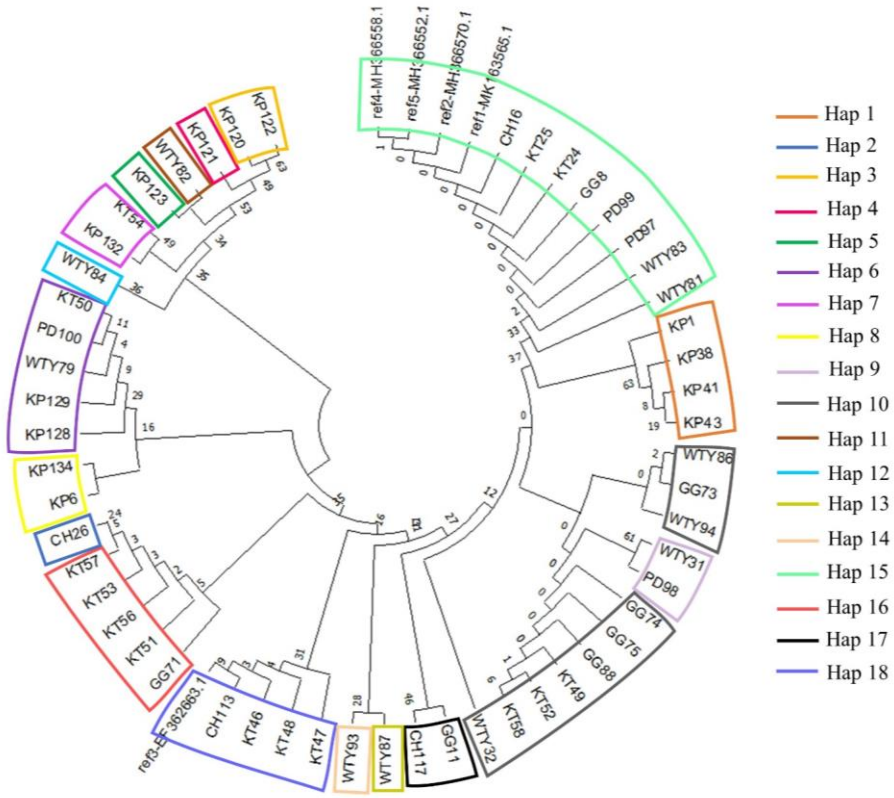

Figure 3. Phylogenetic relationship between haplotypes of COI gene from this study of six native Thai chicken breeds and those of other chicken breeds obtained from GenBank by the Maximum Likelihood method. Note: Six native Thai chickens in this study [KP: Khiew-Phalee, WTY: Leung Hang Khao, PD: Pra Dhu Hang Dam, GG: Thao Thong, CH: Chee and KT: Kai Jae [and other chickens from the NCBI database [CH1-MK163565.1: *Gallus gallus* isolate DC1-1 mitochondrion, CH2-MH366570.1: *Gallus gallus* voucher Kai-Tor011 D-loop, CH3-EF362663.1: *Gallus gallus* isolate bar1-4 D-loop, CH4-MH366558.1: *Gallus gallus* voucher WTYC006 D-loop and CH5-MH366552.1: *Gallus gallus* voucher HL012 D-loop]

Haplotype 10 consisted of 3 breeds including Leung Hang Khao (WTY32, WTY86, WTY94), Thao Thong (GG73, GG74, GG75, GG88) and Kai Jae (KT49, KT52, KT58). Haplotype 11 consisted of Leung Hang Khao (WTY82). Haplotype 12 consisted of Leung Hang Khao (WTY84). Haplotyp 13consisted of Leung Hang Khao (WTY87). Haplotype 14 consisted of Leung Hang Khao (WTY93). Haplotype 15 consisted of 5 breeds comprising Leung Hang Khao (WTY81, WTY83), Pra Dhu Hang Dam (PD97, PD99), Thao Thong (GG8), Chee (CH16) and Kai Jae (KT24 and KT25) and NCBI database (CH1-MK163565.1, CH2-MH366570.1, CH4-MH366558.1 and CH5-MH366552.1). Haplotype 16 consisted of 2 breeds including Thao Thong (GG7) and Kai Jae (KT51, KT53, KT56 and KT57). Haplotype 17 consisted of 2 breeds including Thao Thong (GG11) and Chee (CH117). Haplotype 18 consisted of 2 breeds including Chee (CH113) and Kai Jae (KT46, KT47, KT48) and NCBI database CH3-EF362663.1 (Figure 3).

Evolutionary analysis using Maximum Likelihood Method

From the results of the phylogenetic tree construction of all 6 strains of native Thai chickens compared to the reference data from the NCBI, it was found that haplotypes 1, 3, 4, 5, 6, 7 and 8 could indicate the Khiew-Phalee chicken breed. Haplotypes 1, 4, 5, 6, 7 and 8 comprised the Khiew-Phalee chicken sampled in this research, and haplotype 3 was a Khiew-Phalee chicken strain that was sampled from Phromnoi et al. (2022). Therefore, it could be confirmed that the Khiew-Phalee chicken breed from that area had similar genetic diversity. Haplotype 15 is a haplotype that can indicate the diversity of 5 native chicken breeds (Leung Hang Khao, Pra Dhu Hang Dam, Thao Thong, Chee and Kai Jae) as almost all chicken breeds are in this haplotype. Other haplotypes cannot indicate specific chicken breeds because there are many species distributions within each haplotype.

According to DNA barcode nucleotide sequence analysis to determine mtDNA haplotypes, haplotype diversity (Hd), nucleotide diversity (Pi) and the average number of differences (K) using DnaSP (version 6), the molecular diversity indices of the chicken population in this study were detailed as follows. In terms of Hd, which was inferred from mtDNA sequences (partial D-loop, tRNA-Phe, partial 12S rRNA), it was valued at 0.91. The nucleotide diversity (Pi) of all samples for mtDNA sequences was 0.00504. The average number of k or nucleotide differences from mtDNA gene sequences was 2.69086 (Table 3).

Table 3. Genetic diversity indices of native chickens based on mtDNA barcode

Breeds	N	No.	Hd	Pi	K
Khiew-Phalee	13	1,3,4,5,6,7,8	0.897	0.00696	3.71795
Lueng Hang Khao	11	6,9,10,11,12,13,14,15	0.927	0.00470	2.50909
Pra Dhu Hang Dam	4	6,9,15	0.833	0.00499	2.66667
Thao Thong	7	10,15,16,17	0.714	0.00196	1.04762
Chee	4	2,15,17,18	1.000	0.00343	1.83333
Kai Jae	14	6,7,10,15,16,18	0.857	0.00354	1.89011
Total	53	18	0.917	0.00504	2.69086

Note: N: Sample size, No.: Number of haplotypes, Hd: Haplotype diversity, Pi: Nucleotide diversity, K: Average number of differences

Samples from Khiew-Phalee chickens had the highest nucleotide diversity (0.00696) and an average number of differences (3.71795). Thirteen specimens of Khiew-Phalee chicken showed 7 haplotypes, 0.897 Hd, 0.00696 Pi and 3.1795 K. Eleven specimens of Leung Hang Khao chicken showed 8 haplotypes, 0.927 Hd, 0.00470 Pi and 2.50909 K. Four specimens of Pra Dhu Hang Dam chicken showed 3 haplotypes, 0.833 Hd, 0.00499 Pi and 2.6667 K. Seven specimens of Thao Thong chicken showed 4 haplotypes, 0.714 Hd, 0.00196 Pi and 1.04762 K. Four specimens of Chee chicken showed 4 haplotypes, 1.000 Hd, 0.00343 Pi and 1.8333 K. Finally, fourteen specimens of Kai Jae showed 6 haplotypes, 0.857 Hd, 0.00354 Pi and 1.89011 K, as shown in Table 3.

Discussion

Morphological characterization and DNA barcoding are the two main methods used to identify species. The common DNA barcode gene is used as a molecular marker for DNA barcoding, which increases the speed and accuracy of the identification process for species and organisms (Peng et al. 2019). Khiew-Phalee chickens have morphological differences from other native Thai chicken breeds. In this way, it is possible to understand the specific information and characteristics of Khiew-Phalee chickens.

The bar2 primer was reported in previous studies by Cui et al. (2017) and Innak et al. (2019), who reported the sequence of bar2 was a COI gene barcode. In contrast with the results in this study, the PCR product of bar2 primer is 648 bp as the part of mtDNA control region fragments were BLAST and alimented in NCBI and showed the bar2 DNA barcode gene was partial of D-loop, tRNA-Phe and partial 12S rRNA, loci 712 to 1,358 in *Gallus gallus* complete genome of the mitochondrion. The D-loop is loci 1 to 1231 as the control region, tRNA loci 1232 to 1300 as tRNA-Phe and rRNA loci 1301 to 2276 as 12S ribosomal RNA. A recent study on the COI gene by Peng et al. (2019) identified Danzhou and five other chicken breeds using the COI gene as a DNA barcode to evaluate the differential expression. The primers were designed according to the COI gene sequence of Chinese red raw chicken Cox1 (AP003322) from GenBank. The PCR product size was 648 bp and loci 7015 to 7662 in *Gallus gallus* complete genome of the mitochondrion. Dave et al. (2021) reported that PCR amplification was carried out using primer pairs, namely, BirdF1 and COIbirdR2, for COX I (Lopez and Erickson 2012). Previous studies have presented novel and broadly used primers based on mitochondrial 12S and 16S rRNA genes for Digenea intending to demonstrate their suitability as pathway markers (Ahmed et al. 2022). Mitochondrial 12S and 16S rRNA genes are suitable for trematode molecular identification (Chan et al. 2022) and mitochondrial rRNA gene analysis is a molecular technique that, when combined with bioinformatics, provides a reliable method for the taxonomic classification of animal tissues (Yang et al. 2014). Alternative genetics for order of molecular identifications Plagiorchiida, Echinostomida and Strigeida (Chan et al. 2022).

Other mitochondrial markers such as D-loop (control region) have also been used by various investigators to explore diversity among different native chicken breeds across the world (Miao et al. 2013; Kawabe et al. 2014; Teinlek et al. 2018). A higher number of polymorphic sites defining more haplotypes were reported in native Samar Philippines chickens, native Hungarian chickens, native Egyptian chickens, native chicken breeds of Jiangsu, and chicken breeds native to Korea. These studies showed high genetic diversity with higher haplotype and nucleotide diversity in Samar native Philippines chickens, native Hungarian chickens, native Egyptian chickens, native chicken breeds of Jiangsu and chicken breeds of Korea (Ahmed et al. 2022; Antil et al. 2023). Jarulis et al. (2022) found six specific sites as barcodes for the Sumatran jungle fowl. It has genetic differences from other chickens in the world, so the mtDNA COI gene can be used as a barcode to identify traded red jungle fowl. Jin et al. (2022) studied the full-length D-loop mtDNA sequence of Pingpu Yellow chicken (PYC) breeds and found that the haplotypes were clustered into all three haplogroups (A, B, and C), indicating that PYC may have three maternal origins.

The dominant phenotypic characteristics of Khiew-Phalee chickens were crested comb and red earlobe. The Khiew-Phalee chickens were characterized by their blackish-green color, which was visible around the eyes, beak, neck plumage, back plumage, wing plumage, wing, long curved tail, and back tail, respectively (Phromnoi et al. 2021). Khiew-Phalee chickens and the other breeds had no morphological differences. The morphological traits were difficult to distinguish accurately from others breeds. The Khiew-Phalee chicken population could be evaluated more comprehensively by changing the information on the heterotopic points. In this way, the phylogenetic evaluation was based on mitochondrial DNA (mtDNA) variation in chickens from different areas of the world. However, that

study lacked information on specific breeds and types in Thailand. Mitochondrial DNA control region sequences have frequently been used to assess the diversity and phylogeographic structure of various chicken populations. Recently, these kinds of studies have been able to take advantage of complete chicken mtDNA sequencing (Lasagna et al. 2020) to objectively understand the specific information and characteristics of Khiew-Phalee chickens.

This study measured six breeds of Khiew-Phalee, Thao Thong, Leung Hang Khao, Chee, Pra Dhu Hang Dam and Kai Jae and a total of 53 individual DNA barcode gene sequences, which were found to have 9 different sequence sites containing all transition types of variation. However, the transition mutation of A<->G or T<->C, as shown in Table 1, had a minor effect of single-base substitutions on encoded amino acid types than that of different base substitutions, consistent with the studies of Guo et al. (2017) and Phromnoi et al. (2022). According to Yushi et al. (2007) and Cui et al. (2017), two main sequences of the mtDNA gene of Chinese indigenous breeds were analyzed for genetic diversity using bar1 and bar2 as primers. Hence, the use of bar1 and bar2 as primers for DNA barcoding to identify indigenous chicken breeds was a better choice. Therefore, DNA barcoding could be used to identify and distinguish chicken breeds. However, a complete mtDNA genome is needed to confirm this speculation. Susanti et al. (2018) studied the nucleotide variation of the COI gene in ducks. They found that nine different nucleotides were substitutions and had no deletion or insertion mutation. COI can serve as a barcode if the variation within species is very small.

The phylogenetic tree showed that Khiew-Phalee chickens clustered with Thao Thong, Leung Hang Khao, Chee, Pra Dhu Hang Dam and Kai Jae chickens. The Khiew-Phalee chicken was a low-intensity variety. The bar2 barcode isolated from Khiew-Phalee, Thao Thong, Leung Hang Khao, Chee, Pra Dhu Hang Dam and Kai Jae chicken had similar insertion sequences and positions. This is the result of several recent hybridization or divergence events. The relationship between geographical distribution of all chicken breeds originating from Uttaradit and Phitsanulok provinces showed a close genetic relationship between these chicken breeds (Phromnoi et al. 2022). In addition, Uttaradit and Phitsanulok provinces have relatively similar natural environments. Under the aforementioned circumstances, Khiew-Phalee chickens, which had relatively little selection intensity, inevitably had some form of relationship with the other 5 chicken breeds in Uttaradit and Phitsanulok provinces. However, crossbreeding between the Khiew-Phalee chickens and Pra Dhu Hang Dam, Leung Hang Khao, and Kai Jae chickens showed that there was a genetic exchange between the Khiew-Phalee chickens and Pra Dhu Hang Dam, Leung Hang Khao, and Kai Jae chickens.

Khiew-Phalee chickens are generally used for breeding. It is expected that there will be crossbreeding between domestic and foreign chicken breeds. First, all 5 chicken breeds in this study have large breeding sites in Uttaradit and Phitsanulok provinces due to their proximity. Being geographically close and frequently traded, each breed inevitably has different breeding experiences. Second, the relative conservation of some of the D-loop, tRNA-Phe and 12S rRNA genes is bar2-barcoded, meaning that the genetic differences between the species are relatively minor. In evaluating the breeding potential of Khiew-Phalee chickens and other chicken breeds, it is necessary to consider the differences in morphology, growth efficiency and cytology between these chickens. Molecular characteristics are the basis for the selection and breeding of Khiew-Phalee chickens.

There may be gene exchange between Khiew-Phalee chickens and Pra Dhu Hang Dam, Leung Hang Khao, and Kai Jae chickens in Uttaradit and Phitsanulok provinces. Uttaradit and Phitsanulok have relatively enclosed natural environments, so the proximity between Khiew-Phalee chickens and Pra Dhu Hang Dam, Leung Hang Khao, and Kai Jae chickens is understandable. In addition, the results in the current study indicate that crossbreeding between Khiew-Phalee chickens and Pra Dhu Hang Dam and Leung Hang Khao chickens may have occurred when crossbreeding with Pra Dhu Hang Dam and Leung Hang Khao chickens was introduced in Uttaradit and Phitsanulok provinces.

This research could help promote sustainable agriculture from the genetic diversity of native chickens to domesticated animals (SDG Target 2.5 maintain genetic diversity) (The Sustainable Development Goals Report, 2022). This research unveils the maternal inheritance of Khiew-Phalee chickens and Leung Hang Khao, Pra Dhu Hang Dam and Kai Jae chickens in the provinces of Uttaradit and Phitsanulok, pinpointing them as important genetic resources. However, other molecular markers, including the whole mitochondrial genome or mitochondrial COI gene, should be investigated further to complete the characterization of Khiew-Phalee chickens as well as create a guideline for the conservation and enhancement of chicken breeds and genetic markers to aid in future chicken breeding programs.

In conclusion, it was found that there were gene variations in the mitochondrial DNA barcode of Khiew-Phalee chickens in Uttaradit Province, Thailand when compared with five other Thai native chicken strains as well as reference data from the NCBI. Haplotypes 1, 3, 4, 5, 6, 7 and 8 can indicate the Khiew-Phalee breed of chicken. Haplotype 15 can indicate the diversity of 5 native chicken breeds (Leung Hang Khao, Pra Dhu Hang Dam, Thao Thong, Chee and Kai Jae) as almost all chicken breeds are in this haplotype. However, other haplotypes cannot indicate specific chicken breeds because there are many species distributions within each haplotype. Therefore, mtDNA sequences can be used successfully to develop a phylogenetic tree that shows monophyletic clusters.

ACKNOWLEDGEMENTS

This research was funded by Thailand Science Research and Innovation Project titled "Analysis of Genetic Variations of mitochondrial COI Gene and Protein Profiles in Khiew-Palee Chicken in Uttaradit Province". The

authors would like to thank Uttaradit Provincial Livestock Office, Thailand, for helping in selecting Thai native chickens for the study, the Thai native chicken research network for supplying blood samples and Uttaradit Rajabhat University and Kasetsart University Kamphaeng Saen Campus for laboratory support. The authors declare no conflict of interest.

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