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Genetic diversity and phylogenetic analysis of Khiew-Phalee chickens (Thailand) based on mitochondrial DNA cytochrome b gene sequences

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Abstract. *Phromnoi S, Lertwatcharasarakul P, Phattanakunanan S. 2022. Genetic diversity and phylogenetic analysis of Khiew-Phalee chickens (Thailand) based on mitochondrial DNA cytochrome b gene sequences. Biodiversitas 23: 750-756.* Khiew-Phalee Chickens (*Gallus gallus*) is a Thai indigenous breed, locally recognized for its economic value as an important source of revenue generation. As the Khiew-Phalee chicken cannot be accurately identified by morphological characterization, molecular methods for species identification are generally thought to be complementary and more effective than relying on the method alone. Molecular characterization of Khiew-Phalee Chickens was investigated using mitochondrial DNA (mtDNA) cytochrome b (Cyt b) gene analysis. Partial sequences of the 1,037 bp Cyt b gene were determined on 55 specimens of Khiew-Phalee chickens. The breed's genetic diversity was assessed based on the number of polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity, and average number of differences. In addition, a neighbor-joining (NJ) haplotype tree was constructed based on Kimura's two-parameter model. Haplotype and nucleotide diversity of Khiew-Phalee chickens were 0.597 and 0.00068, respectively. Five haplotypes were identified from 4 polymorphic sites with polymorphism at nucleotides 501, 507, 543, and 1,095. Phylogenetic analysis with the inclusion of the Cyt b gene nucleotide sequences from Khiew-Phalee chickens and native chickens from the GenBank database showed that Khiew-Phalee chickens belonged to *Gallus gallus*. It was closest to the indigenous chicken in South East Asia. The phylogenetic tree indicated the genetic variability of the investigated Khiew-Phalee chicken populations. This study may help future researchers and livestock breeders to design a breeding program based on a better understanding of the genetic diversity and history of indigenous breeds.

Keywords: Haplotype, indigenous chicken, molecular characterization, Thailand, valuable resource

INTRODUCTION

Khiew-Phalee, a fighting cock (*Gallus gallus*), is a native chicken (Indigenous breed) which has its origin in Uttaradit province, the north region of Thailand, and has a long-standing history dating back to 1770 as the fighting cock of Phraya Phichai Dab Hak, a Siamese general serving under King Taksin The Great. The Khiew-Phalee chicken breed has been certified by the Department of Livestock Development as a Thai national domestic animal since 2013 (Biodiversity Research and Development Section 2014). The Khiew-Phalee chicken has been documented in history as a Thai warrior's cock with great fighting skill and a graceful build. It is characterized by its shiny blackish-green plumage and black shaft, clearly visible around the eyes, beak, neck plumage, back plumage, wing plumage, wing, long curve and back tail (Biodiversity Research and Development Section 2014) (Figure 1). The chicken is valuable as a show contestant or for sport and recreation purposes. Phromnoi et al. (2021) preliminarily studied the value of Khiew-Phalee chicken, finding that one Khiew-Phalee egg can be sold for 200-500 baht, a 2-month-old chick for 1500 baht, an 8-month-old mature chick for 3000 baht and an exceptional chick for up to 25000-30000 baht, depending on the exhibited genetic attributes. The main markets are both domestic and international in the Middle East countries. It is one of the country's most valuable resources, being agricultural products that could generate careers and another good source of income for the locals. However, the Khiew-Phalee chicken population showed a tendency to decline and slight acquaintance. The Khiew-Phalee chicken cannot be accurately identified by morphological characterization alone. Molecular methods for species identification are generally considered to be more accurate and efficient.

Figure 1. Khiew-Phalee Chicken from the north region of Thailand. A. Male; B. Female. (Photographs by: Siriwadee Phromnoi)

Genetic diversity affects the existence in the evolutionary process of organisms in everchanging environments (Noro et al. 1998). It is, therefore, necessary to study the reproductive relationships of the same or similar groups of animals. With the growing advance of molecular techniques, nucleotide sequences from mitochondrial DNA (mtDNA) have been used as a biomarker for animal species identification which is beneficial for forensic investigations, animal diversity and evolution studies based on a phylogenetic tree (Noro et al. 1998; Olschewsky and Hinrichs 2021). In mtDNA, the cytochrome b (cyt-b) proteins have been most frequently used for taxonomic and phylogenetic analysis at the species and family level. These genes proved to be useful for estimating divergence in taxa up to the order level in many animal groups. The cyt b gene from the mitochondrial genome contains species-specific information which has been used in phylogeny as well as in forensic investigations and identifying the biological origin of casework specimens (Yacoub et al. 2015; Hartatik et al. 2019).

To our best knowledge, there does not seem to have been studies on the genetic diversity and phylogenetic relationship of the Khiew-Phalee chicken using mtDNA Cyt b gene analysis. Therefore, this study was prompted to gain more insights into the genetic diversity and phylogenetic relationship of the Khiew-Phalee chicken breed using a partial sequence of mtDNA cyt b gene. The present study was thus undertaken to provide useful information for breed improvement and conservation to ensure the existence and growth in the population of the Khiew-Phalee chicken, an important animal that holds significance to Thai local communities. The study could also be a good model for the preservation of the country's heritage resources and the resource development to become economically viable and profitable.

MATERIALS AND METHODS

Study area

The study was conducted in the Uttaradit Province, Thailand, between December 2020 to August 2021 (Figure 2).

Procedures

Ethics statement

Experimental procedures were approved and conducted in accordance with the guidelines of the Animal Ethics Committee, Phibunsongkhram Rajabhat University (Approval reference number: PSRU-(AG)-2020-002).

Collection of blood samples and DNA isolation

Ethylene diamine tetraacetic acid (EDTA) blood specimens were collected by a purposive sampling method from the wing vein of 55 Khiew-Phalee fighting cocks raised in 3 backyard farms, comprised of 14, 9, and 32 from the farms coded as KB, PP, and KS, which have been continuously running Khiew-Phalee chicken farming operations for more than 5 years, respectively in Uttaradit province, Thailand. Twenty microliters of the collected blood were extracted for total DNA using DNeasy®Blood & Tissue Kit (Qiagen, Germany). The DNA extraction was performed according to the manufacturing manual, and 100 microliters of sterile nuclease-free water was used for elution. Eluted DNA samples were stored at -20°C until used.

Amplification of a fragment of mtDNA and nucleotide sequencing

To amplify the partial cytochrome b by polymerase chain reaction (PCR) using specific primers, a forward primer CCATCCAACATCTCCGCATGATGAAA (Branicki et al. 2003) and the r2-3m as reverse primer 5' TGCTTAAGGTTAATTACTGCTG 3' were used (Teinlek et al. 2018). The amplicon of each sample was amplified by Phusion High-Fidelity DNA polymerase (ThermoScientific, Lituania), which had a proofreading activity and analyzed nucleotide sequences by NGS sequencing based on the MiSeq Illumina sequencing platform at BTSeqTM Contiguous Sequencing Service (CELEMICS, South Korea). For those which gave unclear DNA sequences, including double base peak chromatogram and low fragment frequency, the nucleotide sequencing was reperformed using the Sanger sequencing method (CELEMICS, South Korea).

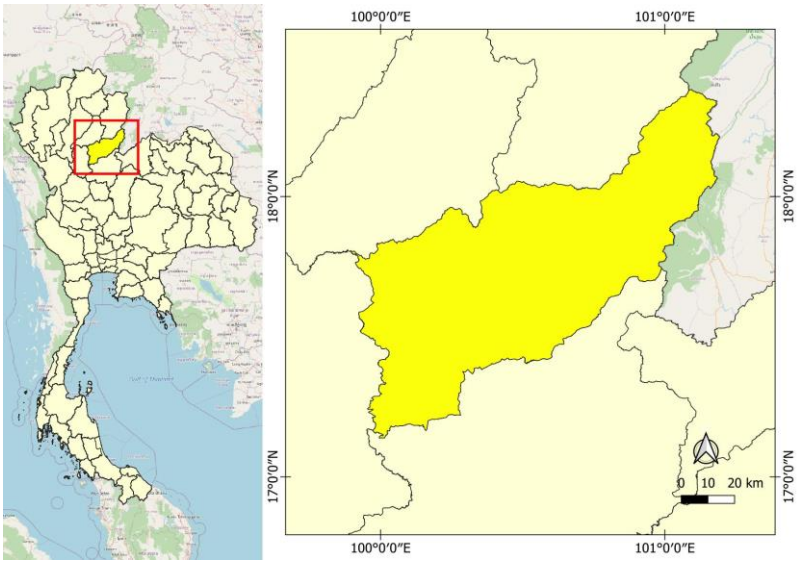

Figure 2. Location of research sites in Uttaradit Province, Thailand

The PCR was conducted in a thermal cycler T100 Thermocycler (BIORAD, USA) with the conditions of 3 minutes initial denaturation at 98°C, followed by 35 cycles, each consisting of 30 s denaturation at 95°C, 30 s annealing at 60°C and 3 min extension at 72°C cycle, and then final extension step at 72°C for 5 minutes. The amplified DNA target was analyzed in 1.5 percent of agarose gel electrophoresis to confirm the length of the amplified fragment. Each amplicon was purified using the FavorPrep GEL/ PCR Purification (Favorgen, Taiwan).

Data analysis

Sequencing analysis and phylogenetic construction

Multiple alignments were performed using the ClastalW program from MEGA version X software among nucleotide sequences based on cytochrome b of Khiew-Phalee (KP) breed and other native chicken breeds obtained from the GenBank database. The specimens from each farm were categorized into a haplotype, and haplotype diversity, nucleotide diversity, the average number of differences, and Tajimas's D test were estimated using DnaSP version 6 (Rozas et al. 2017). The nucleotide sequence reported from the *Gallus gallus* Guangxi chicken (KP681581) was used to determine the nucleotide position of each haplotype. The genetic distance was calculated by the Kimura 2-parameter method. The neighbor-joining (Nj) method was used to generate the phylogenetic tree with 1000 bootstrap replicates using MEGA software (Kumar et al. 2018).

RESULTS AND DISCUSSION

Based on the PCR amplification of 1037 bp Cyt b gene DNA sequence performed on 55 specimens of Khiew-Phalee chickens, the nucleotide sequence was deposited in the database GenBank under the Accession number OK206515-OK206569, when compared with the sequences of the study chickens, different sequences were detected at 4 sites. They were 1 singleton variable site (two variants) at site 501, and 3 parsimony informative sites at sites 507, 543 and 1095. Based on the sequence variations, the chickens were divided into 5 haplotypes, most of which fall into haplotype 1, followed by haplotypes 5, 2, 3, and 4, respectively (Table 1). The haplotypes 1 to 5 were DNA-barcoded as GTCC, GCCC, GTCT, ATCC, and GTTC, respectively. Five haplotypes were identified from 4 polymorphic sites with polymorphism between nucleotides 501, 507, 543, and 1095.

Haplotype 1 was found in 33 samples from all farms: 12 samples from the KB farm, 5 from the PP farm, and 16 from the KS farm. Haplotype 2 was found in 8 samples from KB and KS farms: 2 samples from the KB farm and 6 samples from the KS farm. Haplotype 3 was found in 4 samples, including 3 and 1 samples from PP and KS farms, respectively. One sample from PP farm was a haplotype 4. Haplotype 5 was found in 9 samples from only KS farm (Table 2).

Table 1. Polymorphic sites among haplotypes (The numerical position of each polymorphic site is indicated above each position)

Haplotypes	Numerical position of nucleotide sequence*				N
	501	507	543	1095	
1	G	T	C	C	33
2	●	C	●	●	8
3	●	●	●	T	4
4	A	●	●	●	1
5	●	●	T	●	9

Note: * Position according to complete cytochrome b of chicken breed Guangxi (accession number KP68581) A black dot represents an identical nucleotide compared to haplotype 1.

Table 2. Samples of each farm in haplotypes based on cytochrome b gene

Haplotypes	Farms		
	KB	PP	KS
Haplotype 1	OK206515	OK206529	OK206543
	OK206516	OK206532	OK206544
	OK206517	OK206533	OK206545
	OK206518	OK206535	OK206546
	OK206520	OK206537	OK206547
	OK206521		OK206550
	OK206522		OK206553
	OK206523		OK206554
	OK206524		OK206557
	OK206526		OK206558
	OK206527		OK206560
	OK206528		OK206565
			OK206566
			OK206567
			OK206568
			OK206569
Haplotype 2	OK206519		OK206538
	OK206525		OK206541
			OK206549
			OK206551
			OK206552
			OK206555
Haplotype 3		OK206530	OK206548
		OK206531	
		OK206534	
Haplotype 4		OK206536	
Haplotype5			OK206539
			OK206540
			OK206542
			OK206556
			OK206559
			OK206561
			OK206562
			OK206563
			OK206564

Table 3. Genetic diversity indices of Khiew-Phalee chicken based on cytochrome b gene

Farm	N	No.	Hd	Pi	K	Tajima's D
KB	14	1, 2	0.264	0.00025	0.26374	-0.34144
PP	9	1, 3, 4	0.639	0.00070	0.72222	-0.06382
KS	32	1, 2, 3, 5	0.655	0.00077	0.79435	-0.15336

Note: N: Sample size; No.: Number of haplotype; Hd: Haplotype diversity; Pi: Nucleotide diversity; K: Average number of differences and Tajama's D.

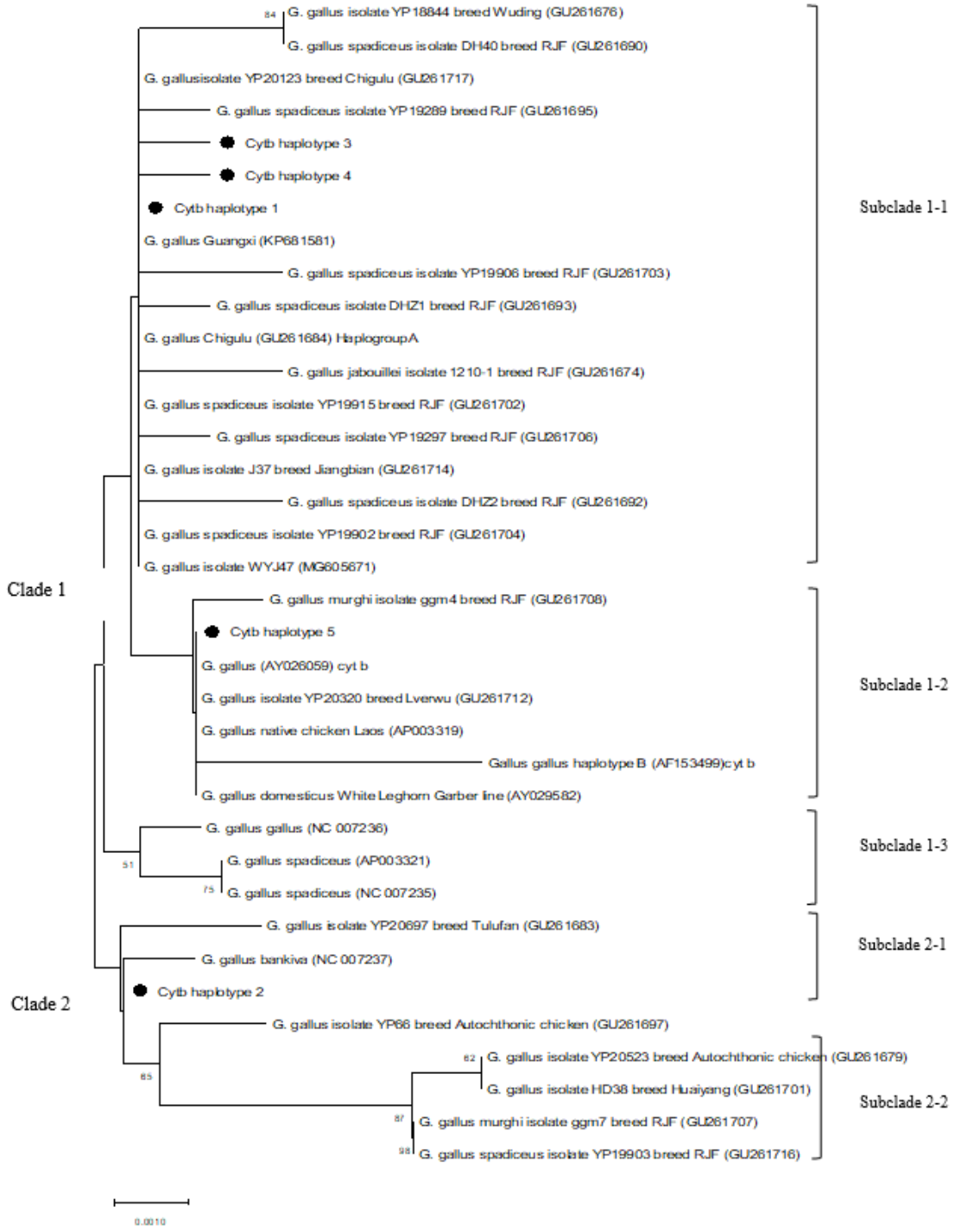

Figure 3. Phylogenetic relationship between haplotypes of Cyt b gene from the Khiew-Phalee breed and those of other chicken breeds obtained from GenBank database by Neighbor Joining (NJ) method. (Black dots represent the data obtained from this study, but otherwise accession numbers in parentheses were from GenBank)

According to Cyt b gene nucleotide sequence analysis to determine mtDNA haplotypes, haplotype diversity (Hd), nucleotide diversity (Pi), the average number of differences (K) and Tajima's D using DnaSP (version 6), the molecular diversity indices of the chicken population in this study were detailed as follows. All samples showed 0.597 haplotype diversity, 0.00068 nucleotide diversity, 0.70572 average number of differences, and -0.42846 Tajima's D. Fourteen specimens from the KB farm showed 2 haplotypes including haplotype 1 and 2, 0.264 haplotype diversity, 0.00025 nucleotide diversity, 0.26374 average number of differences and -0.34144 Tajima's D. Nine specimens from the PP farm showed 3 haplotypes including haplotype 1, 3 and 4, 0.639 haplotype diversity, 0.00070 nucleotide diversity, 0.72222 average number of differences and -0.06382 Tajima's D. Thirty-two specimens from the KS farm showed 4 haplotypes including haplotype 1, 2, 3 and 5, 0.655 haplotype diversity, 0.00077 nucleotide diversity, 0.79435 average number of differences and -0.15336 Tajima's D as shown in Table 3.

Discussion

As the decreasing population of Khiew-Phalee chickens would threaten the genetic diversity of the breed, the study on the genetic diversity of existing Khiew-Phalee chicken populations was conducted to provide guidelines in biodiversity management for future sustainability. This research study was carried out to investigate the molecular biology of the Khiew-Phalee chicken populations in Thailand by the partial nucleotide sequence analysis of the 1037 bp cyt b gene. In this study, 4 mutation sites, identified by the nucleotide sequence variations or polymorphism sites in the cyt b gene, were found as a transition mutation of A<->G or T<->C as shown in Table 1. This is consistent with a study by Guo et al. (2017), stating that transition mutations are generally more likely to occur than transversions, possibly due to the smaller effect of single-base substitutions on encoded amino acid types than that of different base substitutions.

The relatively high values of haplotype diversity, nucleotide diversity, and the average number of differences shown in this study indicated the genetic diversity of the study populations. The Tajima's D neutrality test (Tajima 1989) from the cyt b sequence found that the study populations had a Tajima's D value of -0.42846, which is negative but close to zero. The result indicated that the study populations were in equilibrium. This may be that there has been no rapid population growth of Khiew-Phalee chickens, allowing a gradual change in the genetic structure of the populations to reach equilibrium.

A breakdown of the results by farm showed that the KB farm had lower haplotype diversity, nucleotide diversity, the average number of differences, and Tajima's D than PP and KS (Table 3). This could result from the fact that the KB farm employed a free-range culture. There was once a large population until the outbreak of avian influenza in Thailand in 2008 (Duangjinda et al. 2012), significantly reducing the size of the KB farm's population. The impact of the avian influenza epidemic is closely related to the occurrence of a genetic bottleneck, an evolutionary event in which a large population with high genetic diversity experiences a sharp reduction in size due to temporary environmental events, leading to a reduction in genetic variation of the population (Lv et al. 2015). In contrast to KB, PP and KS used a closed-house system with better farm management, thereby being less affected by the epidemic and not experiencing a sharp decline in the chicken population in the farms. Thus, the haplotype and nucleotide diversity in the two farms were higher than in the KB. However, the negative Tajima's D values for the three farms revealed that the populations of Khiew-Phalee chickens were still in equilibrium.

Based on this study, Khiew-Phalee chickens are regarded as Thai indigenous fowls classified in the species of *G. Gallus*, which most of all are similar to *G. g. spadiceus* from China, Myanmar, and Thailand. This agrees with the studies of Liu et al. (2006); Miao et al. (2013); Pramual et al. (2013); Kawabe et al. (2014); Xie et al. (2016); Suwannapoom et al. (2018), which reported that the indigenous fowls could have originated in China, Myanmar, Laos, Thailand, and India. In addition, the previous study on genetic variations of Thai native fowls, which studied the indigenous chicken breeds and the Thai red junglefowl to investigate genetic variation and evolution history (Phromnoi and Pattanakunanan 2017; Teinlek et al. 2018; Hata et al. 2021). It was found that indigenous chickens mingled with the *Gallus gallus*, junglefowl, the primary ancestor of the domestic chicken, becoming a paraphyletic group. Similarly, the study Khiew-Phalee chickens were all classified as *G. gallus* but found to be genetically diverse as they can be classified into subgroups. The subgroup fragmentation implies the diversity of the chicken population. This may be explained by differences in farming areas. Farms located in distant areas or highland areas have more chance of movement and inbreeding, which may be due to overlap between intra-and interspecific genetic variability. For this reason, further details of molecular genetics should be studied. Nevertheless, this study cannot clearly distinguish Khiew-Phalee native chicken from other native species at molecular levels. This is congruent with a study of Yacoub et al. (2015), finding that although Cyt b nucleotide sequence was an effective tool to discriminate native chickens (Saudi chicken strains) from other species of *Gallus gallus*, it was often used to discriminate interspecies rather than intraspecies.

In this study, specific mitochondrial cyt b gene sequences are not yet an effective approach to examine DNA barcodes for Khiew-phalee chicken breeds. Khiew-phalee chickens were identified and evaluated at the molecular level after morphological studies, which played an essential role in improving the breeding of Khiew-phalee chickens, and the results of the present study would contribute to research on Khiew-phalee chicken breeding strategies in the future. However, the number of specimens involved in this experiment and the sequences generated are not large enough. Therefore, further research is suggested to have more samples for sequencing to validate the application of DNA barcoding. In addition, alternative methods such as a microsatellite marker should be

developed to be used as the genetic marker for identifying Khiew-Phalee native chickens. Future studies on other genes such as D-loop, COI, 16 SrDNA, or ITS (Internal transcribed spacer) both in nuclear DNA (nDNA) and Mitochondrial DNA (mtDNA) should be carried out for conservational advantages and the improvement of their breed available in Thailand.

In conclusion, the genetic diversity analysis of 55 Khiew-Phalee chickens using PCR technique with the PCR products of approximately 1037 bp and the cytochrome b gene sequences in mitochondrial DNA yielded 5 haplotypes obtained from sequence variations at 4 positions: 501, 507, 543, and 1095. Haplotypes 1 to 5 were DNA-barcoded as GTCC, GCCC, GTCT, ATCC, and GTTC, respectively. The most common types found in the chicken breed than any other were haplotypes 1 and 2.

The phylogenetic correlation analysis performed to compare the nucleotide sequences of the investigated Thai Khiew-Phalee chicken with the sequences in the GenBank database using ClustalW found that the chicken breed was classified into 2 clades: clade 1, which was subdivided into 1-2, 1-2 and 1-3, and clade 2 divided into 2 subclades: 2-1 and 2-2. The investigated chickens were classified in both clades 1 and 2: subclade 1-1 (haplotype 1, 3, and 4), subclade 1-2 (haplotype 5), and subclade 2-1 (haplotype 2). Khiew-phalee chicken populations are generally most closely related to populations that are geographically near. They could have shared common ancestors with the indigenous chickens from South East Asia (China, Myanmar, Indonesia, Laos), India, and Japan.

This present study shows the practicality of mitochondrial DNA cyt b gene sequences for the analysis of diversity and genetic relationships of the Khiew-phalee chicken. However, the study results in the Khiew-phalee chicken contribute to the knowledge of genetic information of Thai native chicken. Due to the crisis of a sharp decline in the size of the Khiew-phalee native chicken populations, our data would be useful for further planning to be undertaken by the Department of Livestock Development of Thailand, to establish an effective breeding program and conservation plan for this species.

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