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Allelic diversity of *Blec2* gene in indigenous and local chickens and red junglefowl in Thailand: Implications for disease resistance

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ABSTRACT

Although selective breeding significantly enhances production traits in commercial chickens, it often compromises their immune function. Indigenous chickens, however, typically exhibit strong disease resistance. The major histocompatibility complex plays a critical role in the adaptive immune responses to pathogens in chickens. The *Blec2* gene in the MHC-B region, which encodes a putative natural killer cell receptor, is a promising candidate that influences the early immune responses. Little, however, is known about polymorphisms of this gene in indigenous and local chicken breeds or red junglefowl in Thailand. In this study, polymorphisms in a partial fragment of exon 4 and intron 3 of *Blec2* were examined using targeted next-generation sequencing and genetic diversity analyses. Fourteen alleles and nine single nucleotide substitutions were identified; these included both silent and missense mutations, which may influence immune function. Notably, one allele, *Blec2*TH2*, referred to as haplotype 21, is reported to be strongly associated with resistance against the H5N1 virus. Purifying selection alongside stochastic processes were also observed in this gene fragment, indicating a strong potential for disease resistance. By contrast, *Blec2*TH13* allele referred to as haplotype 13, which was previously reported to correlate with 100% mortality rate for avian influenza, was detected in Nin Kaset breed. The study findings indicate the existence of diverse immune response mechanisms in indigenous and local chickens and red junglefowl in Thailand. These findings provide valuable insights that should be relevant for information for developing breeding programs using marker-assisted selection to enhance the immune resilience of commercial poultry stocks.

Introduction

Climate change poses a significant threat to poultry production by increasing the risk of disease outbreaks (Godde et al., 2021). Diversity in domestic chickens is derived from wild ancestors, such as the red junglefowl (*gallus*), through genome alterations via domestication and artificial selection over thousands of years. Recent selection programs have improved desirable traits, which has likely led to the rapid erosion of genetic variations within and among chicken breeds, strains, and lines (Chebo et al., 2022). Genetic profiles of domestic chicken and red

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junglefowl populations are crucial for their long-term preservation, driven by natural selection pressures, and maintenance of disease resistance and poultry production sustainability. Thailand abounds with red junglefowl and domestic chicken resources with high genetic polymorphisms (Hata et al., 2021; Singchat et al., 2022; Budi et al., 2023; Wattanadilokcahtkun et al., 2023; Wongloet et al., 2023; Tanglertpaibul et al., 2024). Domestication of chickens is believed to have started in South and Southeast Asia from wild red junglefowl according to the hypothesis, Thailand is one of the original sites of domestication (Peters et al., 2016, 2022). Thus, red junglefowl from Thailand may contain the original genetic profile of domestic chickens; therefore, indigenous and local chickens in Thailand are regarded as a valuable source of genetic variation. For instance, the H5N1 avian influenza virus outbreak, which occurred in many Asian countries from November 2003 to March 2004, resulted in substantial mortality of infected humans and devastating losses to the poultry industry. However, certain populations of indigenous chickens in Thailand exhibit remarkable resistance to this highly pathogenic virus (Boonyanuwat et al., 2006). This suggests that selection and crossbreeding have affected the genomic regions associated with disease resistance traits in indigenous and local chickens in Thailand. Thus, leveraging genomic technologies and experimental infections is required to identify disease resistance genes. Challenges, however, include large-scale infections of avian influenza and Newcastle disease (Van et al., 2020; Olaniyan et al., 2024), warranting greater awareness of the importance of domestic chicken populations, which may be achieved through their genetic characterization and conservation. The genetic variation in indigenous and local chickens and red junglefowl would be accessible for current and future breeding initiatives, including enhancement of disease resistance.

Differences in immune responses are linked to diverse mediator proteins, such as major histocompatibility complex (MHC) molecules and antibodies, as well as environmental factors, such as housing and nutrition. At the molecular level, they are attributed to the difference of functional efficiency and diversity of immune-mediator molecules. The MHCs are encoded by a tightly linked gene family that help discriminate between self and non-self cells and regulate adaptive immune responses to parasites and diseases (Eizaguirre et al., 2012; Eimes et al., 2013). In higher vertebrates, MHCs have been extensively studied because of their diversity across species and direct link with disease resistance and other biological functions (Boonyanuwat et al., 2006; Eimes et al., 2013). MHCs encompass a cluster of class I (*MHC-I*) and class II (*MHC-II*) genes, which are critical for recognizing intracellular and extracellular pathogens (Blum et al., 2013; He et al., 2023). Both *MHC-I* and *MHC-II* possess a two-domain peptide-binding region (encoded by exons 2 and 3 in *MHC-I* and exon 2 in *MHC-IIA* and *MHC-IIB*) that directly interacts with antigenic peptides. The MHC region also harbors genes that encode proteins involved in antigen processing, such as transporters associated with antigen processing (TAPs), in which linkage pattern between antigen processing genes and antigen-presenting genes are various between species (Lankat-Buttgereit & Tampé, 2002; He et al., 2023). Comparative studies across diverse taxa have highlighted significant variations in the size, number, and organization of MHC loci (Kelley et al., 2005; Boonyanuwat et al., 2006). In chickens, *MHC-I, MHC-IIB*, and other associated genes are clustered within a compact core region on chromosome 16, spanning less than 100 kb. By contrast, *MHC-IIA* is believed to be located approximately 5.6 centimorgans away from the core MHC region (includes *MHC-I* and *MHC-IIB* genes) on the same chromosome (Salomonsen et al., 2003; Kaufman, 2014). Domestic chickens possess only two classical class I genes, *BF1* and *BF2* (Kaufman & Venugopal, 1998; Wallny et al., 2006). The MHC-B region encompasses *BG, Blec*, and *BTN* genes (Shiina et al., 2004, 2006; Chaves et al., 2011). Additionally, two MHC class IIB genes are present in domestic chickens (Kaufman, 1999). One gene significantly associated with disease resistance is *Blec2*, a putative natural killer (NK) cell receptor. *Blec2* is associated with resistance to Marek's disease in domestic chickens (Kelley & Trowsdale, 2005; Rogers & Kaufman, 2008). *Blec2* (also known as *B-NK*) is considered a strong candidate that affects early immune responses to the Marek's disease virus (Shiina et al., 2007). *Blec2* may recognize MHC class I alleles encoded by the Y region. A functional inhibitory signaling motif is present in the cytoplasmic tail domain of *Blec2*, which is expressed in NK cells (Rogers et al., 2005; Shiina et al., 2006). These observations highlight the role of MHC molecules in disease resistance of domestic chickens, including both indigenous and local chickens in Thailand. However, data on *Blec2* polymorphisms related to disease resistance in domestic chickens and red junglefowl are lacking, despite indigenous chickens infected with infectious diseases exhibit lower mortality rates (Buranawit & Laenoi, 2021).

Genetic diversity studies conducted on indigenous and local chicken breeds in Thailand, as well as on red junglefowl, have consistently shown high levels of genetic variation, based on microsatellite genotyping and mitochondrial d-loop sequence analysis (Hata et al., 2021; Singchat et al., 2022; Budi et al., 2023; Wattanadilokcahtkun et al., 2023; Wongloet et al., 2023; Tanglertpaibul et al., 2024). If variation of the *Blec2* gene in red junglefowl is revealed and accessible, the maintenance of genetic diversity becomes more vital for combating infectious diseases and adapting to environmental changes, and would provide an untapped source of genetic information for improving agricultural diversity in chicken breeds, strains, and lines. It is hypothesized that red junglefowl possess great diversity in the *Blec2* gene, which is influenced by pathogen inventories related to geographical locations and habitat types. Artificial selection may further reduce the allelic diversity in indigenous and local chickens in Thailand, while red junglefowl may maintain their ancestral alleles. The current genetic composition of indigenous and local chickens in Thailand could possibly contain only a small fraction of the genetic diversity carried in their wild ancestors, red junglefowl, many of which are endangered or extinct. This study was aimed at comprehensively evaluating the polymorphisms in a partial fragment of exon 4 and intron 3 of the *Blec2* gene using targeted next-generation sequencing and genetic diversity analyses in 11 natural populations of red junglefowl and 25 indigenous and/or local chicken breeds in Thailand. The findings of this study are expected to aid conservation and development of breeding programs to enhance the immune capabilities of poultry using marker-assisted selection.

Materials and methods

Sample collection and DNA extraction

Blood samples were collected from 25 populations of 16 indigenous and local chicken breeds and 11 populations of red junglefowl in Thailand. Further information on the samples used in this study is presented in Table S1. Permission was granted by the farm owners and all chickens were immediately released into the same area after sample collection. Whole blood specimens were collected from the brachial wing vein using Vacuette® 21-gauge needles and then transferred into vials containing 5 mM EDTA (Greiner Bio-One, Kremsmünster, Austria) and stored at 4 °C until use. Genomic DNA was extracted following the standard salting-out protocol described by Supikamolseni et al. (2015). DNA quality and quantity were assessed using electrophoresis on 1 % agarose gel and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). All experimental procedures were approved by the Kasetsart University Animal Experiment Committee (Approval No: CKU63-SCI-02, ACKU63-SCI-022, ACKU65-SCI-017, ACKU65-SCI-021, ACKU66-SCI-001, and ACKU66-SCI-004) and were carried out in accordance with the Regulations on Animal Experiments at Kasetsart University and the ARRIVE guidelines (https://arriveguidelines.org/).

Polymerase chain reaction amplification and illumina™ short-read sequencing

The partial fragment of exon 4 and intron 3 of the *Blec2* gene, which

show polymorphism across Galliformes, were amplified via polymerase chain reaction (PCR) using the primer set pcBlec2F (5′-GACAGAGCAGGCAGGCAGCA-3′) and pcBlec2r (5′-GGGCTGCAACCACCCCAGTT-3′) (Eimes et al., 2013). To the 5′-end of each forward primer, specific 8 bp individual barcode sequences were added (Macrogen Inc., Seoul, Korea). Each 15 μL of PCR mixture consisted of 25 ng DNA template and 1 × Apsalagen buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM primers, and 0.5 U Taq polymerase (Apsalagen Co., Ltd., Bangkok, Thailand). The PCR was run with an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were detected by electrophoresis on a 1 % agarose gel. Each sample was run in triplicates to avoid false allele amplification. Ninety-two samples amplified per pool set with each barcode primer were pooled into six pool sets and sent for paired-end short-read sequencing on an Illumina NovaSeq™ 6000 platform (Novogene Co., Ltd., Singapore).

Sequence quality control and sequence demultiplexing

The paired end 250-basepair reads were evaluated using FASTQC version 0.12.0 to ensure a quality score above 20 (Andrews, 2010). The paired-end reads were merged and demultiplexed to obtain individual sequences within each pool, followed by isolation and filtering of individual amplicon sequences and assignment of *Blec2* allele numbers per individual using AmpliSAS tools (Sebastian et al., 2016). To account for low reads and potential artifacts, the minimum amplicon depth was set to 100. The maximum number of alleles per individual was set to six, to account for possible duplications in *Blec2* genes (He et al., 2022). The degree of change (DOC) filtering parameter was used to estimate true allelic numbers based on sequencing depth (Lighten et al., 2014). Default settings were used for all other parameters.

Individual alleles were checked for similarity to other nucleotide sequences in the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLASTn. All sequences were aligned and translated into amino acid sequences using Geneious Prime version 2024.0.5 (https://www.geneious.com/). No stop codons were observed in the coding region of partial fragment of exon 4 from this study. The allele sequences found in this study were deposited in the DNA Data Bank of Japan (https://www.ddbj.nig.ac.jp/, accessed on December 2, 2024) (accession numbers: LC853225–LC853238).

Phylogenetic analysis of the Blec2 gene

A phylogenetic tree was used to visualize the evolutionary history of the *Blec2* alleles in chickens; it was constructed using MrBayes version 3.2.6, based on Bayesian inference (Ronquist & Huelsenbeck, 2003). ModelFinder (Kalyaanamoorthy et al., 2017) implemented in IQ-TREE was used to determine the best-fit substitution model, based on the lowest Bayesian information criteria (BIC) value. The MCMC process was performed simultaneously for the four chains over one million generations. After stabilizing the log-likelihood value, sampling was performed every 100 generations to obtain 10,000 trees and to generate a majority-rule consensus tree with mean branch lengths. All the initial sample points were discarded during the burn-in period. Partial *Blec2* gene sequences from different global chicken breeds were obtained by retrieving partial fragments of exon 4 and intron 3 of the *Blec2* gene from the NCBI database using BLASTn. The phylogenetic tree was visualized using the Interactive Tree of Life (iTOL) version 5 online (Letunic & Bork, 2021). Genetic differentiation between populations was assessed using Principal Coordinate Analysis (PCoA), which was based on allelic frequency data. Pairwise genetic distances were calculated with the R package "poppr" in R version 4.3.2, which was used to generate the distance matrices. A haplotype network for *Blec2* alleles was inferred using the median-joining algorithm, which was implemented in PopART v1.7. Networks were constructed from aligned nucleotide sequences, in which each circle represents a haplotype and circle size was proportional to its frequency. Mutational steps were indicated by lines and dashes, which allowed visualization of haplotype sharing and specificity among populations (Bandelt et al., 1999).

Genetic diversity and selection analysis

Genetic diversity was estimated by calculating the number of alleles (N_a) and nucleotide diversity (π) using DnaSP version 6.12 (Rozas et al., 2017). The average amount of synonymous (d_S) and nonsynonymous (d_N) substitutions per site was estimated using the Nei–Gojobori's method (Nei & Gojobori, 1986) with Jukes–Cantor correction, and then a Z-test was carried out to assess the d_N/d_S ratio (ω). A ratio close to 1 indicates neutrality, ω values >1 indicate positive selection, whereas ω values <1 indicate purifying selection. To further investigate the possible selection of the *Blec2* gene, neutrality tests based on the frequency spectrum (eg., Tajima's *D*, Fu's *F*, Fu and Li's *F**, or Fu and Li's *D**) were performed using DnaSP version 6.12 (Rozas et al., 2017).

Protein structure prediction and visualization

The amino acid sequences of the partial exon 4 of the *Blec2* gene were used to predict the tertiary (3D) structure of the protein using I-TASSER (Yang & Zhang, 2015). The resulting model was visualized in BIOVIA Discovery Studio (Dassault Systèmes, San Diego, CA, USA), and its quality was assessed using PROCHECK (Laskowski et al., 1993) based on the Ramachandran plot. The modeled structures were aligned and compared with the reported Lectin like natural killer cell surface protein from Uniprot database (https://www.uniprot.org/).

Results

Polymorphism of the fragments of exon 4 and intron 3 of Blec2 gene in indigenous and local chicken breeds and red junglefowl in Thailand

The nucleotide sequences of 155 bp partial fragments covering exon 4 and intron 3 of *Blec2* were obtained by short-read sequencing, which contained α-helix-turn-β-strand and coil motifs (Fig. 1). A total of 14 alleles of the *Blec2* gene, containing nine variable sites, were identified. Among these, eight alleles were newly identified, and one allele (*Blec2*TH2*) was identical to the reference sequence (accession number AB268588). The *Blec2*TH1* allele was the most common in indigenous and local chicken breeds and red junglefowl in Thailand. *Blec2*TH9, Blec2*TH11, Blec2*TH13*, and *Blec2*TH14* were exclusively identified in indigenous and local chicken breeds. *Blec2*TH9* was detected only in FL-CRRBC, *Blec2*TH11* in LPK, *Blec2*TH13* in BLWF and BLBF, and *Blec2*TH14* in BLBF. Additionally, *Blec2*TH8* and *Blec2*TH10* were specific to red junglefowl (Table S2), whereas *Blec2*TH8* was exclusive to *G. gallus spadiceus* populations derived from the Khao Kho and Chiang Mai Zoo, whereas *Blec2*TH10* was found in *G. gallus spadiceus* population from the Songkhla Zoo. Compared with the reference sequences, the 14 alleles exhibited six mutational sites in the exon, consisting of three silent mutations and three missense mutations, along with three sites in the intron, which included two transversions ($T > C$) and a transition ($A > G$) (Table 1 and Table S3; Fig. 1). The number of alleles per individual ranged from one to five, with a mean π value of 0.018 (Table 2). In particular, indigenous and local chicken breeds exhibited allele numbers between two and five, with a mean π value of 0.018, whereas red junglefowl carried two to four alleles, with a mean π value of 0.017. Bayesian phylogenetic tree revealed a polyphyletic pattern, indicating a lack of distinct clustering based on breed or geographic origin within indigenous and local chickens and red jungle fowl in Thailand (Fig. 2). PCoA based on *Blec2* allele data showed that the first and second principal coordinates (PCo1 and PCo2), which accounted for 31.6 % and 26.6 % of the total genetic variation respectively, explained the majority of variation (Fig. S1). Local chicken and red junglefowl populations,

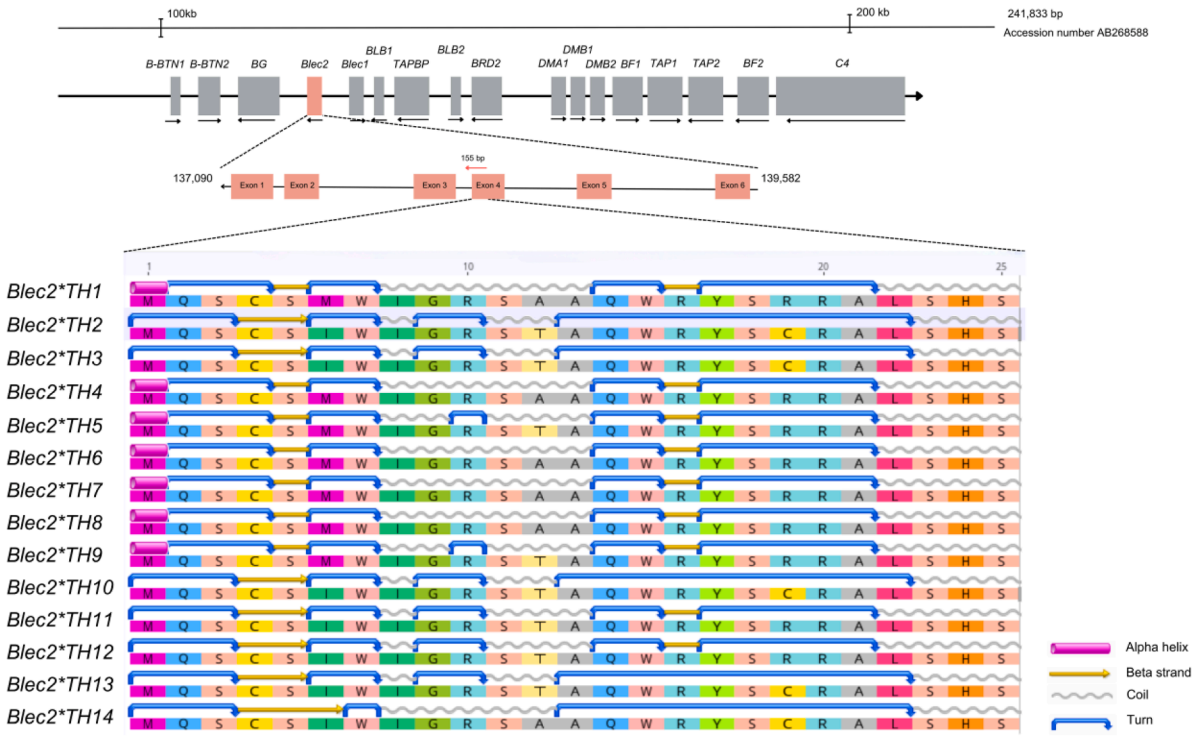

Fig 1. Amino acid sequences of *Blec2* alleles and prediction of secondary structures of protein. The protein secondary structure was predicted for amino acid residues in the partial fragment of exon 4 of the *Blec2* gene in Thai indigenous and local chicken breeds and red junglefowl.

Table 1
Variable sites of *Blec2* gene alleles found in this study.

Allele	Accession number	Intron 3			Exon 4					
		Nucleotide position*								
		138,104	138,118	138,141	138,165	138,168	138,171	138,187	138,195	138,208
Reference sequence	AB268588	T	T	A	C	A	T	A	A	T
*Blec2*TH1*	LC853225	.	.	G	T	C	G	G	.	C
*Blec2*TH2*	LC853226	.	.	.	.	.	.	.	.	.
*Blec2*TH3*	LC853227	.	.	.	.	C	.	.		.
*Blec2*TH4*	LC853228	.	.	G	T	C	G	G	G	C
*Blec2*TH5*	LC853229	.	.	G	T	C	G	.	.	C
*Blec2*TH6*	LC853230	.	C	G	T	C	G	G	G	C
*Blec2*TH7*	LC853231	.	.	.	T	C	G	G	G	C
*Blec2*TH8*	LC853232	.	.	.	T	C	G	G	.	C
*Blec2*TH9*	LC853233	.	C	G	T	C	G	.	.	C
*Blec2*TH10*	LC853234	C	.	G	.	C	.	.	.	.
*Blec2*TH11*	LC853235	.	.	G	.	C	.	.	.	C
*Blec2*TH12*	LC853236	.	.	.	.	C	.	.	.	C
*Blec2*TH13*	LC853237	.	.	.	.	C	.	.	G	.
*Blec2*TH14*	LC853238	.	.	.	.	C	.	G	.	.

* Nucleotide position based on reference sequence (accession number AB268588)

which were plotted across the ordination, did not form distinct clusters by origin or geographic area. A haplotype network for *Blec2* revealed 14 haplotypes, of which *Blec2*TH1, Blec2*TH3*, and *Blec2*TH5* were the most common; several haplotypes (*Blec2*TH9, Blec2*TH10, Blec2*TH13*, and *Blec2*TH14*), which occurred at low frequency, were located on separate branches (Fig. 3).

Selection analyses of indigenous and local chicken breeds and red junglefowl in Thailand based on the Blec2 gene

Evidence for the selection of the *Blec2* gene was revealed by the Z-test in most indigenous and local chicken breeds and red junglefowl populations in Thailand (Table 3). The ω value for the *Blec2* gene could not be calculated due to the absence of d_S in most of the population of indigenous and local chicken breeds in Thailand, except for the LHK2-F, LHK3-O, and WZ populations. The ω values for the LHK2-F, LHK3-O, and WZ populations were 0.553, 0.551, and 0.361, respectively, indicating that purifying selection was involved in the present genetic structures of these populations. For red junglefowl, ω values of the *Blec2* gene could not be calculated due to the absence of d_S in all populations. Neutrality tests for the *Blec2* alleles revealed variations in Tajima's *D*, Fu and Li's *D**, and Fu and Li's *F* values for indigenous and local chicken breeds and red junglefowl in Thailand (Table 4). Tajima's *D* values, ranging from −0.849 to 2.482, were not significant for all populations, except for the LHK3-O, MHS-CLRBC, MHS-F, MHS-MLRBC, SD1, SD2, *G gallus spadiceus* (Huai Sai), *G. galus gallus* (Chiang Mai Zoo), and DT-U populations. The Fu and Li's *D** ranged from −0.969 to 1.352 and was insignificant, except for the CF-MLRBC population. The Fu and Li's *F**, ranging from

Table 2
Diversity of nucleotide sequences of the *Blec2* gene.

Breed/ red junglefowl subspecies	Population	N	N_a	π	*SD*
Lueng Hang Khao	Phitsanulok Farm	19	5	0.018	0.001
	Phitsanulok Panyanukun School	16	5	0.019	0.001
	Phitsanulok	10	3	0.017	0.003
Pradu Hang Dam	Phitsanulok 1	10	3	0.013	0.003
	Chiang Mai	13	5	0.017	0.003
Chee Fah	Chiang Rai	10	2	0.016	0.003
	Mae Hong Son	10	3	0.020	0.004
Fah Luang	Chiang Rai	9	4	0.014	0.004
	Mae Hong Son	10	2	0.021	0.002
Mae Hong Son	Chiang Mai	6	4	0.018	0.001
	Mae Hong Son Farmer	4	4	0.018	0.001
	Mae Hong Son Provincial Livestock office	15	4	0.020	0.002
Khaew Paree	Phitsanulok	10	3	0.013	0.003
Lao Pa Koi	Lamphun	9	2	0.011	0.002
Dong Tao	Lopburi	5	3	0.011	0.005
	Udon Thani	10	2	0.019	0.001
Samae Dam	Uthai Thani Provincial Livestock office	6	3	0.019	0.002
	Sanhawat Farm	3	4	0.020	0.002
Nin Kaset White	Lopburi	10	2	0.016	0.003
Nin Kaset Black	Lopburi	10	4	0.018	0.003
Betong	Lopburi	14	3	0.019	0.002
Wein Chang	Udon Thani	10	2	0.010	0.005
Shiang Hai	Chiang Mai	10	2	0.015	0.003
Rose	Chiang Mai	10	2	0.015	0.004
Decoy	Phitsanulok, Sukhothai, Chiang Mai	5	2	0.011	0.005
G. gallus	Khon Kaen Zoo	8	4	0.019	0.001
	Huai Sai	3	3	0.021	0.004
	Chanthaburi	10	2	0.017	0.002
	Roi Et	10	3	0.012	0.002
	Songkhla Zoo	10	3	0.018	0.002
	Si Sa Ket	10	3	0.005	0.001
G. gallus spadiceus	Huai Sai	10	3	0.015	0.002
	Khao Kho	10	4	0.016	0.002
	Sa Kaew	10	4	0.018	0.002
	Songkhla Zoo	6	2	0.017	0.003
	Chiang Mai Zoo	9	2	0.016	0.002
Indigenous and local breeds		-	-	0.018	-
Red junglefowl		-	-	0.017	0.001
Overall		-	-	0.018	-

N, number of samples; N_a, number of maximum alleles per individuals; π, nucleotide diversity; *SD*, standard deviation.

−1.01 to 1.909, was also statistically insignificant, except for the LHK2-F, LHK3-O, MHS-F, MHS-MLRBC, SD1, SD2, BTG, *G. gallus spadiceus* (Huai Sai), *G. galus gallus* (Chiang Mai Zoo), and DT-U populations.

Multiple sequence alignment of Blec2 amino acid residues and prediction and visualization of their protein structures

The amino acid sequences of the *Blec2* gene were similar to those of the reference sequence of *G. gallus* (accession number: AB268588). The sequence identities ranged from 94.8 % to 100 % and the query cover values ranged from around 80 % to 100 %. The alleles were found for the nucleotide sequences at positions 138,153 to 138,228 in exon 4, encoding 26 amino acids at positions 73 to 98, which are located in the killer cell lectin-like receptor domain. Homology analysis of protein sequences showed that the amino acid sequences in exon 4 of *Blec2* gene alleles had 80–100 % homology with the sequences of Korena native chicken, Huxu, Nicobari, Ghangus, Cornell, and White Leghorn chicken breeds, with 80–100 % query coverage. The 3D structures of predicted amino acids were characterized into three model sets as (A) whole loop-coil, (B) half loop-coil and (C) alpha helix (Fig S2). Only 3D structures from the *Blec2*TH01, Blec2*TH04, Blec2*TH06, Blec2*TH7*, and *Blec2*TH08* exhibited stable / full alpha helix form composed of 25 amino residues. Ramachandran plot analysis of model set A revealed that each amino residue was placed at the most favored region of the full alpha helix structure with 95.5 % of core residue value. By contrast, the 3D structures of model set B and set C were located in the most favored region with 36.4 % to 77.3 % of core residue value, which depended on the amino acid composition of deduced sequence of each allele. These models were all matched with 'lectin like natural killer cell surface protein' (accession number: B5BSM1). The peptide residues were potentially located on C-type lectin domain (Fig S3 and S4).

Discussion

In this study, 14 alleles of the *Blec2* gene were identified in indigenous and local chicken breeds and red junglefowl in Thailand, including eight novel alleles. Analysis of the partial *Blec2* gene fragments revealed nine variable sites, suggesting a higher Single nucleotide polymorphism (SNP) allelic frequency than previously reported (Shiina et al., 2007; Hosomichi et al., 2008; Yuan et al., 2021). Despite this, low nucleotide diversity ($\pi < 0.05$) was observed with shared alleles among chicken populations, likely reflecting the constraints that limit variation within *Blec2*. Such low allelic variation of *Blec2* may significantly have substantial implications for the maintenance of immune function and disease resistance (Hosomichi et al., 2008). By contrast, microsatellites revealed high genetic diversity in indigenous and local chicken breeds and red junglefowl in Thailand (Hata et al., 2021; Singchat et al., 2022; Budi et al., 2023; Wattanadilokcahtkun et al., 2023; Wongloet et al., 2023; Tanglertpaibul et al., 2024). This suggests that these *Blec2* alleles may have been subjected to selective pressure. Most indigenous and local chicken breeds and red junglefowl in Thailand exhibit an absence of synonymous mutations ($d_S = 0$), similar to the inbred UCD001 line which was established from red jungle fowl in Malaysia in the late 1930s (Shiina et al., 2007). This pattern reflects strong purifying selection against synonymous codon changes, resulting in very low or no synonymous substitutions in essential genes (Zhu et al., 2014). Such strong purifying selection in immune-related genes have been observed in populations exposed to high pathogen loads and recurrent disease outbreaks (Mukherjee et al., 2009). This is supported by previous studies that regarded the *Blec2* gene as critical for resistance to diseases, including Marek's disease (Kelley &Trowsdale, 2005; Rogers & Kaufman, 2008). By contrast, it is generally believed that MHC variations are maintained by balancing selection (Hess & Edward, 2002), as shown in previous studies on red junglefowl from Vietnam (Nguyen-Phuc et al., 2016). This disparity may be attributed to different selection mechanisms acting on various genes within the MHC (Rogers & Kaufman, 2008). The Tajima's *D* neutrality test yielded an average of 2.337, which was statistically significant ($p < 0.05$), suggesting balancing selection, consistent with the findings in red junglefowl (Nguyen-Phuc et al., 2016). Conversely, the average of Fu and Li's *D* was 0.238, showing no significant deviation from neutrality, indicating that genetic variations in indigenous and local chicken breeds and red junglefowl populations in Thailand may result from stochastic processes, such as genetic drift and/or selection, as observed in chickens, white-tailed eagles, and other birds (Lighten et al., 2017; Minias et al., 2019; Castro-Rojas et al., 2024; Liu et al., 2024). Bias may have been introduced by small sample sizes in several chicken breeds, which could be attributable to sampling error (Guo et al., 2023). Further investigation, which should include larger sample sizes drawn from diverse populations within each breed, is required to determine whether selection has contributed to the observed genetic variation. Sample sizes per breed were limited, which is a common constraint in studies of wild or endangered populations. Artefacts can be reduced by applying deep sequencing with stringent filtering, which allows reliable inference of allelic diversity even when sample sizes are small (Newhouse et al., 2015; Liu et al., 2020).

A total of 51 alleles have been identified worldwide based on partial fragments of exon 4 and intron 3 of the *Blec2* gene. Phylogenetic analysis revealed polyphyletic patterns within the MHC region, indicating that

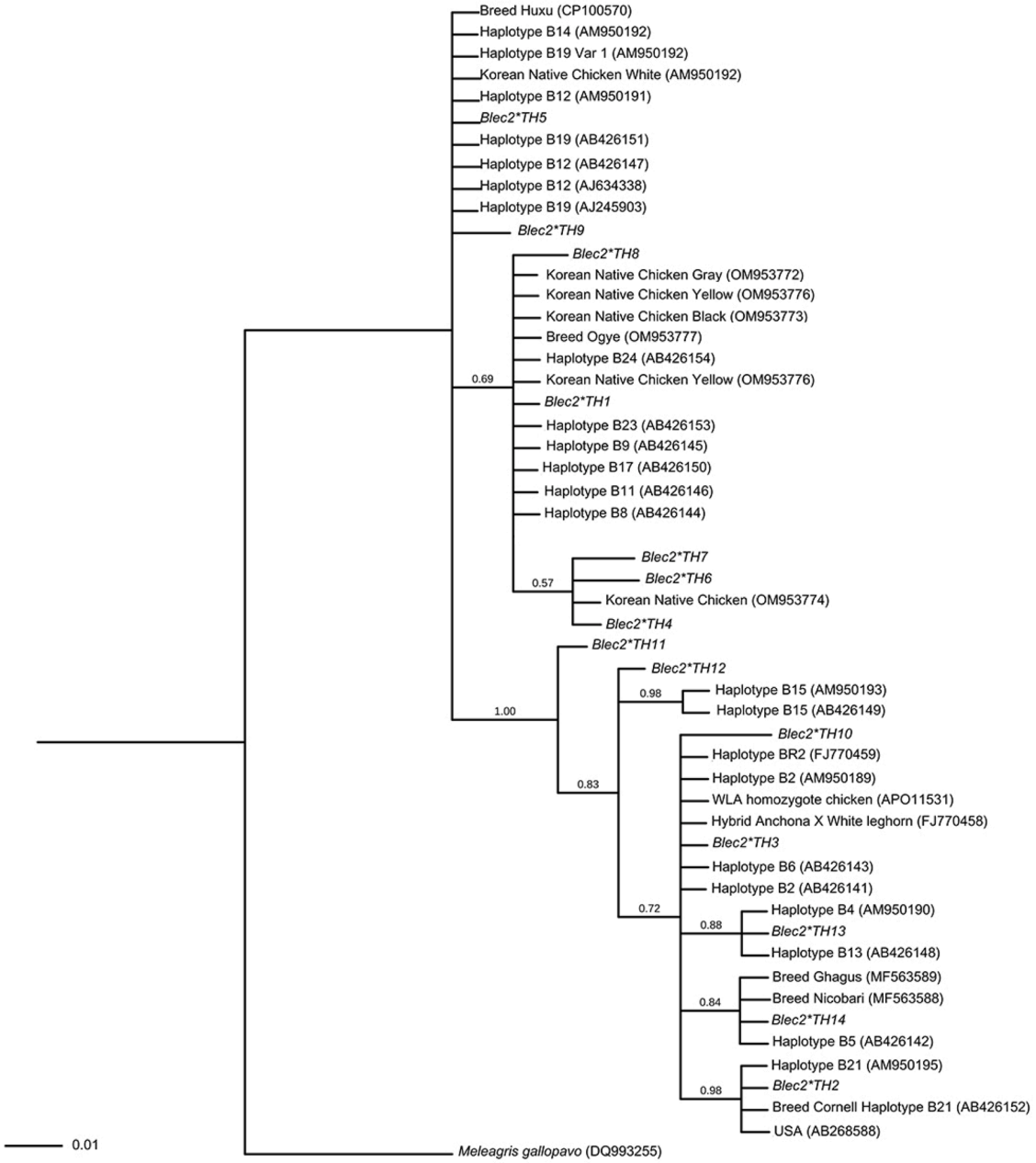

Fig 2. Maximum likelihood phylogenetic tree of *Blec2* gene alleles for Thai indigenous and local chicken breeds and red junglefowl. The values above the branches represent bootstrap values.

there are no specific alleles that cause changes in phylogenetic lineages (Fig. 2). In this study, indigenous and local chicken breeds and red junglefowl in Thailand shared six alleles (*Blec2*TH1, Blec2*TH2, Blec2*TH3, Blec2*TH4, Blec2*TH13*, and *Blec2*TH14*) with several chicken breeds from different regions of the world, which suggests that these alleles are common in chickens. *Blec2*TH2* referred to as haplotype 21, which is known to exhibit complete resistance to the H5N1 virus (Boonyanuwat et al., 2006), is widely distributed in indigenous and local chicken breeds and red junglefowl populations in Thailand, suggesting a potential link of this allele to H5N1 virus resistance. According to the "Red Queen Arms Theory", beneficial novel variant alleles correlated with susceptibility to immune diseases may be introduced to the gene pools of populations through mutations, recombination or migrations, followed by positive selection (Lighten et al., 2017). This is reflected in the phylogenetic tree that placed the *Blec2*TH2* allele in newly emerged clades, indicating that this allele may have been introduced into the gene pools of indigenous and local chicken breeds and red junglefowl in Thailand and maintained through these processes, thereby providing immune protection and promoting positive selection. By contrast, haplotype 13, which shares the *Blec2*TH13* allele found in the Nin Kaset populations (BLBF and BLWF), exhibited 100 % mortality rates for avian influenza (Boonyanuwat et al., 2006). *Blec2*TH13*, which was placed in a considerably new clade, may have undergone purifying selection owing to its non-beneficial immunity after being introduced into the gene pools of the Nin Kaset populations. This suggests a diverse mechanism of disease resistance in indigenous and local chickens in Thailand, potentially attributable to their broader environmental ranges and exposure to various pathogens, compared with that in red junglefowl (Gul et al., 2022). However, the possibility of incidental occurrence is undeniable.

Notably, eight new alleles, *Blec2*TH5, Blec2*TH6, Blec2*TH7, Blec2*TH8, Blec2*TH9, Blec2*TH10, Blec2*TH11*, and *Blec2*TH12,* were

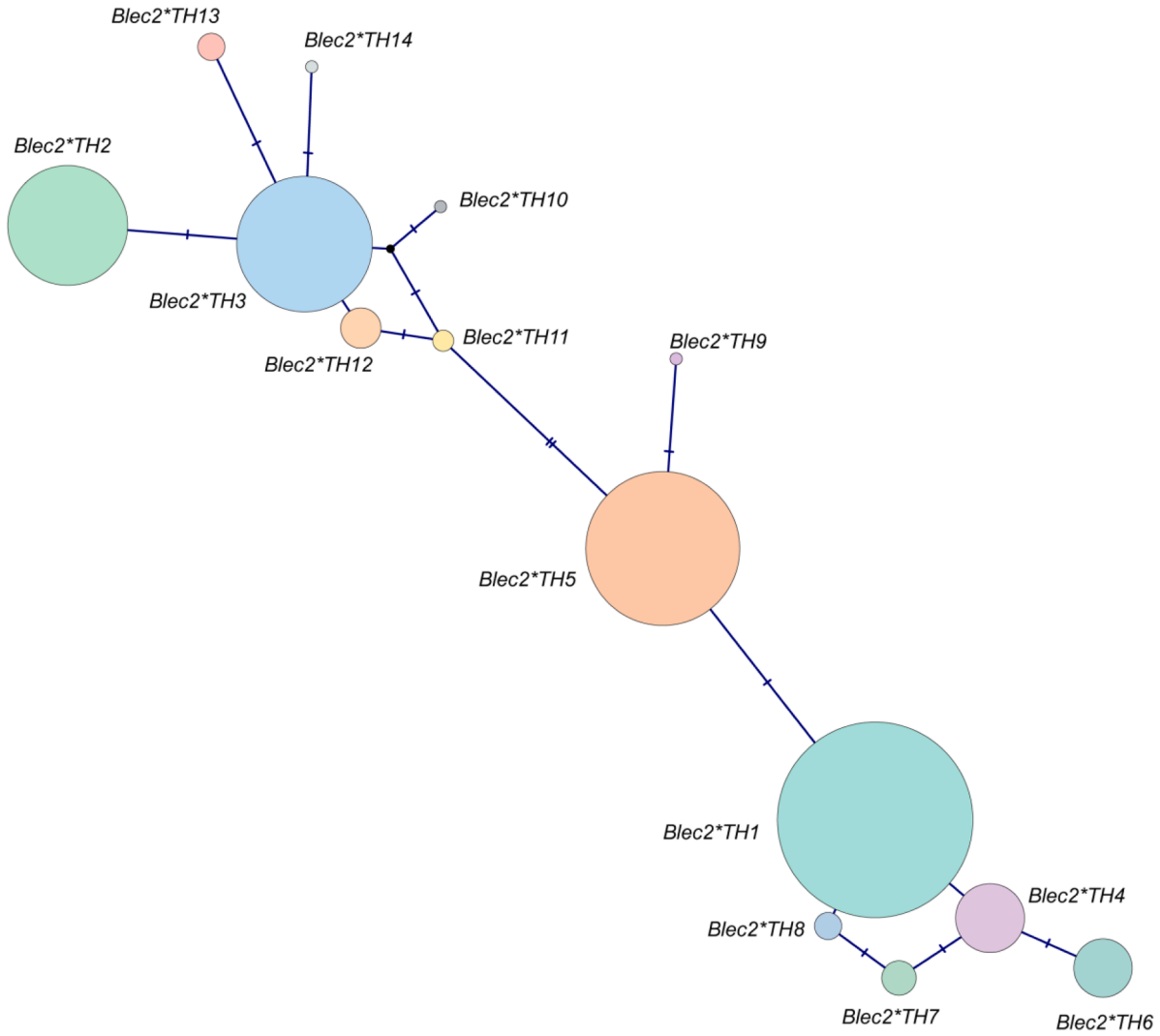

Fig 3. Haplotype network of the Blec2 gene identified in Thai indigenous and local chicken breeds and red junglefowl. Each circle represents a haplotype, with the circle size proportional to its frequency in the populations. Lines connecting haplotypes indicate mutational steps, and short bars on the branches represent inferred mutational events.

identified in this study. Based on the phylogenetic analysis, *Blec2*TH5* may be considered an ancestral allele, which is supported by its grouping with haplotypes 12 and 19 that are regarded as ancestral (Hosomichi et al., 2008). The widespread distribution of *Blec2*TH5* in indigenous and local chicken breeds and red junglefowl in Thailand suggests that this allele may confer an adaptive advantage for disease resistance in tropical climates (Budi et al., 2024; Gaczorek et al., 2024). Two alleles, *Blec2*TH8* and *Blec2*TH10*, were specific to three populations of red junglefowl; *Blec2*TH8* was present in the populations from the Khao Kho and Chiang Mai Zoo and *Blec2*TH10* in the population from the Songkhla Zoo. This suggests that these alleles may be ancestral and retained at low frequencies within the populations because of genetic drift or the retention of divergent lineages resulting from large population sizes and incomplete lineage sorting. Four alleles, *Blec2*TH9, Blec2*TH11, Blec2*TH13* and *Blec2*TH14*, were observed exclusively in the indigenous and local chicken breeds, suggesting their potential adaptation to environment in Thailand (Bonneaud et al., 2006; Eizaguirre et al., 2012). In particular, *Blec2*TH9* detected infrequently in FL-CRRBC had two silent mutations and two missense mutations compared with the reference sequence (AB268588). These missense mutations resulted in amino acid changes from isoleucine to methionine and cysteine to arginine at amino acid positions 138,171 and 138,208, respectively. *Blec2*TH13*, found specifically in BLBF and BLWF, exhibited two silent mutations (138,168A>*C* and 138,195A>*G*), whereas *Blec2*TH14*, which is specific to BLBF, exhibited only one silent mutation (138,168A>*C*). Amino-acid substitutions caused by missense mutations were predicted to alter secondary structure, which may disturb the function of lectin-like natural killer cell surface proteins and reduce their ability to recognize the dominantly expressed *MHC* class I molecule (*BF2*), potentially affecting disease-resistance mechanisms (Rogers & Kaufman, 2008; Straub et al., 2013). *Blec2*TH11*, however, was found exclusively in the LPK chicken, a fighting chicken breed subjected to rigorous artificial selection (Wattanadilokcahtkun et al., 2023). This allele exhibited a silent mutation (138,168A>*C*) and a missense mutation (138,208T>*C*), resulting in a change from cysteine to arginine compared with the reference sequence. The breed specificity of these allele variations is likely due to a combination of natural and artificial selection in the process of domestication. Notably, the LPK chicken also possesses specific allele variations of the genes associated with thermotorelance (Budi et al., 2024), which emphasize the impact of selective breeding. Remarkably, the 14 alleles identified in this study exhibited three distinct protein structural conformations: (i) whole loop-coil, (ii) half loop-coil, and (iii) alpha helix. The common alleles (*Blec2*TH1* and *Blec2*TH4*) exhibited the most stable structure. By contrast, the *Blec2*TH2* allele referred to as haplotype 21 and the *Blec2*TH13* allele associated with 100 % mortality rate for the avian

Table 3
Rates of synonymous (d_S) and nonsynonymous (d_N) substitutions in the nucleotide sequences of the *Blec2* gene.

Breed/ red junglefowl subspecies	Population	d_N	d_S	ω (d_N / d_S)	*Z*-test	
					Z-score	*p*-value
Lueng Hang Khao	Phitsanulok Farm	0.028 ±0.015	0.050 ±0.036	0.553	−0.584	0.584
	Phitsanulok Panyanukun School	0.028 ±0.014	0.050 ±0.036	0.551	−0.558	0.578
	Phitsanulok	0.036 ±0.015	0.000 ±0.000	-	2.548	0.012
Pradu Hang Dam	Phitsanulok 1	0.032 ±0.013	0.000 ±0.000	-	2.553	0.012
	Chiang Mai	0.040 ±0.016	0.000 ±0.000	-	2.559	0.012
Chee Fah	Chiang Rai	0.030 ±0.010	0.000 ±0.000	-	2.290	0.020
	Mae Hong Son	0.040 ±0.020	0.000 ±0.000	-	2.680	0.010
Fah Luang	Chiang Rai	0.029 ±0.012	0.000 ±0.000	-	2.376	0.019
	Mae Hong Son	0.048 ±0.020	0.000 ±0.000	-	2.404	0.018
Mae Hong Son	Chiang Mai	0.041 ±0.018	0.000 ±0.000	-	2.360	0.020
	Mae Hong Son Farmer	0.047 ±0.021	0.000 ±0.000	-	2.287	0.024
	Mae Hong Son Provincial Livestock office	0.042 ±0.017	0.000 ±0.000	-	2.346	0.021
Khaew Paree	Phitsanulok	0.028 ±0.013	0.000 ±0.000	-	2.157	0.033
Lao Pa Koi	Lamphun	0.026 ±0.013	0.000 ±0.000	-	1.793	0.076
Dong Tao	Lopburi	0.025 ±0.014	0.000 ±0.000	-	1.986	0.049
	Udon Thani	0.040 ±0.020	0.000 ±0.000	-	2.250	0.030
Samae Dam	Uthai Thani Provincial Livestock office	0.045 ±0.020	0.000 ±0.000	-	2.308	0.023
	Sanhawat Farm	0.046 ±0.021	0.000 ±0.000	-	2.217	0.029
Nin Kaset White	Lopburi	0.036 ±0.016	0.000 ±0.000	-	2.363	0.020
Nin Kaset Black	Lopburi	0.038 ±0.015	0.000 ±0.000	-	2.442	0.016
Betong	Lopburi	0.050 ±0.020	0.000 ±0.000	-	2.530	0.010
Wein Chang	Udon Thani	0.012 ±0.009	0.033 ±0.025	0.361	−0.782	0.436
Shiang Hai	Chiang Mai	0.032 ±0.016	0.000 ±0.000	-	1.997	0.048
Rose	Chiang Mai	0.036 ±0.014	0.000 ±0.000	-	2.639	0.009
Decoy	Phitsanulok, Sukhothai, Chiang Mai	0.020 ±0.010	0.000 ±0.000	-	2.150	0.030
G gallus	Khon Kaen Zoo	0.042 ±0.018	0.000 ±0.000	-	2.258	0.026
	Huai Sai	0.045 ±0.018	0.000 ±0.000	-	2.565	0.012
	Chanthaburi	0.040 ±0.020	0.000 ±0.000	-	2.260	0.030
	Roi Et	0.023 ±0.012	0.000 ±0.000	-	2.083	0.039
	Songkhla Zoo	0.036 ±0.016	0.000 ±0.000	-	2.407	0.018
	Si Sa Ket	0.014 ±0.011	0.000 ±0.000	-	1.328	0.187
G gallus spadiceus	Huai Sai	0.033 ±0.017	0.000 ±0.000	-	2.111	0.037
	Khao Kho	0.035 ±0.015	0.000 ±0.000	-	2.153	0.033
	Sa Kaew	0.039 ±0.017	0.000 ±0.000	-	2.335	0.021
	Songkhla Zoo	0.036 ±0.018	0.000 ±0.000	-	1.930	0.056
	Chiang Mai Zoo	0.040 ±0.020	0.000 ±0.000	-	2.050	0.040
Indigenous and local breeds		0.035 ±0.016	0.005 ±0.004	6.562	1.972	0.085
Red junglefowl		0.035 ±0.016	0.000 ±0.000	-	2.135	0.045
Overall		0.035 ±0.016	0.004 ±0.003	9.438	2.022	0.073

Table 4
Neutrality test for the *Blec2* gene sequences.

Breed/ red junglefowl subspecies	Population	Tajima's *D*	Fu and Li's *F*	Fu and Li's *D*
Lueng Hang Khao	Phitsanulok Farm	1.972ns	1.732*	1.257ns
	Phitsanulok Panyanukun School	2.440*	1.909*	1.229ns
	Phitsanulok	0.460ns	0.803ns	0.788ns
Pradu Hang Dam	Phitsanulok 1	0.186ns	1.133ns	1.296ns
	Chiang Mai	1.400ns	1.532ns	1.285ns
Chee Fah	Chiang Rai	0.775ns	1.334ns	1.304ns
	Mae Hong Son	1.210ns	1.511ns	1.352*
Fah Luang	Chiang Rai	−0.489ns	0.555ns	0.845ns
	Mae Hong Son	1.601ns	0.703ns	0.211ns
Mae Hong Son	Chiang Mai	2.235*	1.203ns	0.450ns
	Mae Hong Son Farmer	2.289*	1.782*	1.240ns
	Mae Hong Son Provincial Livestock office	2.225*	1.751**	1.326ns
Khaew Paree	Phitsanulok	0.522ns	0.624ns	0.547ns
Lao Pa Koi	Lamphun	0.606ns	0.542ns	0.420ns
Dong Tao	Lopburi	−0.793ns	−1.010ns	−0.969ns
	Udon Thani	2.188*	1.747*	1.253ns
Samae Dam	Uthai Thani Provincial Livestock office	2.482**	1.837**	1.265ns
	Sanhawat Farm	2.018*	1.695*	1.346ns
Nin Kaset White	Lopburi	1.259ns	1.449ns	1.259ns
Nin Kaset Black	Lopburi	0.846ns	0.922ns	0.777ns
Betong	Lopburi	1.928ns	1.714*	1.275ns
Wein Chang	Udon Thani	−0.313ns	0.973ns	1.260ns
Shiang Hai	Chiang Mai	1.746ns	1.554ns	1.206ns
Rose	Chiang Mai	0.496ns	1.251ns	1.313ns
Decoy	Phitsanulok, Sukhothai, Chiang Mai	−0.849ns	−0.936ns	−0.936ns
G gallus	Khon Kaen Zoo	1.998ns	1.165ns	0.562ns
	Huai Sai	0.369ns	0.281ns	0.219ns
	Chanthaburi	1.509ns	0.971ns	0.594ns
	Roi Et	0.248ns	0.547ns	0.562ns
	Songkhla Zoo	1.379ns	1.529ns	1.304ns
	Si Sa Ket	1.040ns	1.067ns	0.896ns
G gallus spadiceus	Huai Sai	2.139*	1.676*	1.186ns
	Khao Kho	1.577ns	1.548ns	1.244ns
	Sa Kaew	1.003ns	0.469ns	0.157ns
	Songkhla Zoo	1.171ns	0.955ns	0.738ns
	Chiang Mai Zoo	2.008*	1.637*	1.214ns
Indigenous and local breeds		2.732*	2.074**	1.133ns
Red junglefowl		1.570ns	1.013ns	0.443ns
Overall		2.337*	1.237ns	0.238ns

*, $p < 0.05$.
**, $p < 0.02$; ns, not significant.

influenza virus (Boonyanuwat et al., 2006) clustered together and adopted partial loop-coil conformations, suggesting reduced structural stability. The changes of protein stability may promote adaptive flexibility, allowing for diverse pathogen resistance mechanisms. Less stable structures, being less constrained, may undergo significant conformational shifts, exploring alternative functional states for adaptation, though these shifts may also have detrimental effects (Mayerguz et al., 2007; Burke & Elber, 2012; Gilson et al., 2017). There, however, is no certainty for these speculation on the functions of proteins with altered structures because only short amino acid sequences were analyzed. Future studies with complete protein sequences and functional assays are needed to validate these speculations.

The higher number of alleles observed in indigenous and local chicken breeds than in red junglefowl in Thailand may be attributed to reduced selective pressure from relaxed purifying selection, allowing for greater allelic accumulation. Indigenous and local chicken breeds may exhibit diverse disease resistance mechanisms owing to some alleles that are preserved in the populations, which may not be advantageous for red junglefowl (Wattanadilokcahtkun et al., 2023). In contrast to the indigenous and local breeds, the absence of synonymous mutations in all red junglefowl populations supports this speculation. Another possible explanation is the founder effect, in which specific alleles may have been dominant or amplified in the population, contributing to the higher genetic diversity observed in local chicken breeds, such as FL-CRRBC, BLWF, and BLBC, which were originally derived from Chinese black-bone chickens (Budi et al., 2023). Alternatively, the lower number of alleles observed in red junglefowl suggests that the reliance on other immune mechanisms, such as robust innate defenses, reduces the need for a costly adaptive immune response mediated by MHC (Gangoso et al., 2012; Minias et al., 2019). A higher number of alleles in indigenous chickens than in red junglefowl has also been reported for microsatellite markers (Granevitze et al., 2009). The observed patterns of higher alleles number exhibited by indigenous and local chicken breeds together with the absent of synonymous mutations in red junglefowls may be coincidental; therefore, functional analysis of these alleles found in this study are needed to obtain more conclusive evidence of its role in chicken immunity.

Conclusion

Understanding the genetic diversity of MHC-B genes has been recognized as essential for maintaining adaptive traits related to disease resistance in chicken breeds. This study focused on the genetic diversity of *Blec2* in indigenous and local chicken breeds and red junglefowl in Thailand, highlighting the need for conservation efforts to prevent genetic dilution caused by homogenization in commercial chicken breeds and to develop breeding strategies for enhancement of their immune capabilities. Despite the low genetic diversity observed in the Blec2 gene, indigenous chicken breeds retain substantial economic potential due to their unique adaptive traits and cultural value, and they remain important reservoirs for maintaining broader genetic variation. This study identified a low polymorphism in a partial fragment of exon 4 and intron 3 of *Blec2*, with *Blec2*TH2* allele that is widely distributed among indigenous and local chickens in Thailand. This allele referred to as haplotype 21 is associated with H5N1 influenza virus resistance. By contrast, the *Blec2*TH13* allele referred to as haplotype 13 found in the Nin Kaset chicken breeds has been associated with 100 % mortality rate for avian influenza. Given the growing threat from climate change and emerging infectious diseases, further research on the disease resistance of indigenous and local chickens is essential. Additionally, functional studies are required to better understand the functional significance of these alleles. The present study provides a basis for considering *Blec2* as a potential genetic marker for improving immune functions in chickens through molecular marker-assisted selection, although confirmation through association analyses and validation in broader chicken populations is still needed.

Ethical statement

All experimental procedures were approved by the Kasetsart University Animal Experiment Committee (Approval No: CKU63-SCI-02, ACKU63-SCI-022, ACKU65-SCI-017, ACKU65-SCI-021, ACKU66-SCI-001, and ACKU66-SCI-004) and were carried out in accordance with the Regulations on Animal Experiments at Kasetsart University and the ARRIVE guidelines (https://arriveguidelines.org/).

Data availability

The allele sequences found in this study were deposited in the DNA Data Bank of Japan (DDBJ) (https://www.ddbj.nig.ac.jp/, accessed on December 2, 2024) (accession numbers: LC853225– LC853238). All genotyping data are available from the Dryad Digital Repository Dataset (https://www.sci.ku.ac.th/scbp/; https://doi.org/10.5061/dryad.hhmgqnkm0, updated on March 3rd, 2025).

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Declaration of generative AI and AI-assisted technologies

Generative AI tools (OpenAI ChatGPT, GPT-4o) were used solely for the purposes of language refinement and editing. Additionally, the manuscript was further reviewed and edited by a professional scientific editing service to ensure clarity and adherence to academic publishing standards. The authors take full responsibility for the content of the published article.

CRediT authorship contribution statement

Trifan Budi: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Worapong Singchat:** Methodology, Investigation, Formal analysis. **Nivit Tanglertpaibul:** Methodology, Investigation, Formal analysis. **Thanyapat Thong:** Methodology, Investigation, Formal analysis. **Thitipong Panthum:** Methodology, Investigation, Formal analysis. **Aingorn Chaiyes:** Methodology, Investigation, Formal analysis. **Narongrit Muangmai:** Methodology, Investigation, Formal analysis. **Orathai Sawatdichaikul:** Validation, Methodology, Investigation, Formal analysis. **Darren K Griffin:** Methodology, Investigation, Formal analysis. **Prateep Duengkae:** Methodology, Investigation, Formal analysis. **Yoichi Matsuda:** Supervision, Methodology, Investigation, Formal analysis. **Kornsorn Srikulnath:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vas.2025.100532.

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