Molecular Characteristics and Evolutionary Relationships of Toll-Like Receptor (TLR21) of Indonesian KUB-1 Chicken

Suryohastari RRB[1,2], Sumarsono SH[1], Rachman EAG[1]

[1]*School of Life Sciences and Technology, Bandung Institute of Technology, 10 Ganesha Street, Bandung, 40132, West Java, Indonesia*
[2]*Department of Biology, Faculty of Sciences and Technology, Syarif Hidayatullah State Islamic University Jakarta, 95 Ir. H. Juanda Street, Cempaka Putih, East Ciputat, South Tangerang, 15412, Banten, Indonesia*
E-mail: sonyheru@sith.itb.ac.id

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ABSTRAK

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Pada hewan vertebrata, sistem imun memiliki *Toll-like receptor* (TLR) untuk mengenali pola molekul terkait patogen tertentu. Pada aves, TLR21 dikenal sebagai homolog fungsional dari TLR9 pada mamalia. Penelitian ini bertujuan untuk mengetahui karakteristik molekuler TLR21 pada ayam Kampung Unggul Balitbangtan (KUB-1) dan hubungan kekerabatannya secara evolusinaris. Gen *TLR21* diperoleh dari ekstraksi RNA ovarium dan disintesis menjadi cDNA melalui transkripsi terbalik. Kami menemukan bahwa cDNA *TLR21* ayam KUB-1 memiliki nukleotida sepanjang 3504 pasang basa (pb), yang termasuk *open reading frame* (ORF) 2823 pb, yang mengkodekan protein putatif dari 940 asam amino (aa). Protein TLR21 ayam KUB-1 hasil deduksi, dibangun dari 720 aa pada domain ekstrasel yang mengandung 20 LRR, 23 aa pada domain transmembran, dan 141 aa pada domain intrasel, serta memiliki berat molekul 107 kDa. TLR21 ayam KUB-1 masing-masing memiliki homologi sekitar 99%, 76%, dan 43% terhadap TLR21 ayam galur lain, unggas non-ayam, dan ikan. Sepanjang evolusi pada TLR21 ayam, karakter molekulernya masih dilestarikan sehingga TLR21 ayam KUB-1 tidak jauh berbeda dengan Broiler. Evolusi TLR21 telah terjadi pada itik dan angsa, meskipun fungsinya tetap sama. Secara evolusioner, TLR21 *Gallus gallus*_KUB-1 yang termasuk dalam Famili TLR11, telah terdiferensiasi dari TLR21 ikan dan membentuk hubungan parafiletik dengan TLR21 pada *Anser cygnoides* dan *Anas platyrhynchos*.

Kata Kunci: *Gallus gallus*, KUB-1, Ovarium, Transkripsi Terbalik, TLR21

ABSTRACT

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In vertebrates, the immune system has several Toll-like receptors (TLRs) to recognize specific pathogen-associated molecular patterns (PAMPs). In aves, TLR21 is known to be a functional homolog of TLR9 in mammals. This study aimed to determine the molecular characteristics of TLR21 in KUB-1 chicken, a superior breed of Indonesian local chicken, and their evolutionary relationship. The TLR21 gene was obtained from the ovarian RNA extraction and synthesized into cDNA by reverse transcription. We found that the cDNA sequence of the TLR21 of KUB-1 chicken has a length of 3504 bp, including a 2823 bp open reading frame (ORF), which encodes a putative protein of 940 amino acids (aa). The deduced KUB-1 chicken TLR21 protein consisted of 720 aa in the extracellular domain containing 20 LRRs, 23 aa in the transmembrane region, and 141 aa of Toll-IL-1 receptor in the intracellular domain, and had a molecular weight of 107 kDa. The KUB-1 chicken TLR21 had homology of about 99%, 76%, and 43% with other TLR21 from other breeds of chicken, non-chicken poultry, and fish, respectively. Its molecular character was conserved throughout the evolution of TLR21 in chicken, so the KUB-1 chicken was not much different from the broiler. However, there has been an evolution in duck and goose, although the function is still the same. Evolutionarily, *Gallus gallus*_KUB-1 TLR21, which belongs to the TLR11 Family, has differentiated from fish TLR21 and formed a paraphyletic relationship with TLR21 in *Anser cygnoides* and *Anas platyrhynchos*.

Key Words: *Gallus gallus*, KUB-1, Ovary, Reverse Transcription, TLR21

INTRODUCTION

The sensory receptors capable of connecting the environment with the immune system have been studied since the discovery of the Toll-like Receptors (TLRs) family. After the discovery of human TLRs, it was immediately followed by the characterization of mammalian TLRs, and since then, members of that family have also been found in birds (Keestra et al. 2013; Juul-Madsen HR. 2013). TLRs play a role in innate immunity with their function as pattern recognition receptors (Song et al. 2015) and are type I transmembrane glycoproteins (Zhang et al. 2016). Functional studies of TLRs detected their ability to

recognize various Pathogen-associated molecular patterns (Ruan et al. 2015). The high level of evolutionarily conserved TLRs in vertebrates has allowed their phylogenetic classification into groups of receptors that respond to similar ligands. They can be divided into cell surface TLRs (TLR1, 2, 4, 5, and 15) and intracellular TLRs (TLR3, 7, and 21), which are localized in the endosome that recognizes nucleic acids from bacteria and viruses (Keestra et al. 2013; Nawab et al. 2019).

In chicken, TLR3, 4, 5, and 7 are close orthologs of TLRs in other vertebrates (Świderská et al. 2018), and TLR1, 6, and 10 in mammals are replaced by TLR1La/b and TLR 2a/2b from chicken are both orthologs of a single TLR2 in mammals (St. Paul et al. 2013). Avian TLR21 is an ortholog of the TLR21 of teleost (Lai et al. 2019). Cells transfected with the chicken TLR21 gene can recognize CpG-ODN, which plays the same role as TLR9 in mice (Yeh et al. 2013). Furthermore, TLR21 protein generally consists of 965-986 amino acids (aa), which includes a series of leucine-rich repeats (LRRs) in the extracellular domain (ECD), a transmembrane (TM) region, and a Toll-IL-1 receptor (TIR) on the intracellular domain (ICD) in the cytosol (Wu et al. 2018). The individual LRR consists of 20-30 aa (Matsushima et al. 2007). Based on ECD architecture, vertebrate TLRs are classified into eight families (Family 1/3/4/5/7/11/13/15), and TLR21 is a member of Family 11 (Wang et al. 2016).

Each TLR family is characterized by its LRR motifs that can be divided into highly conserved (LRRh) and variable (LLRv). The LRRh consists of LxxLxLxxNxL or LxxLxLxxCxxL motifs, where L residues at positions 1, 4, 6, and 11 participate in the hydrophobic core (Matsushima et al. 2015). The chicken TLR21 has a "typical type" (LRR-Typ) at LRR2/3/14/15/15/17 (Li et al. 2018). The LRR domain adopts an arc shape that plays a role in pathogen recognition and binding. Most LRR structures have a cap consisting of two or four cysteines to protect the hydrophobic core (Mokhtari et al. 2021). The TIR of chicken TLR21 contains 125-200 residues required for protein-protein interactions and the conserved sequence at residue 778-943 (Wu et al. 2018). The TIR transmits signals to the cytosol by recruiting the adapter protein responsible for producing pro-inflammatory cytokines (Wicherska-pawłowska et al. 2021). The genetics of chicken breeds significantly influence the production of pro-inflammatory cytokines in response to the challenge of *Salmonella* (Kaiser et al. 2022).

Salmonella can contaminate chicken eggs (Popa & Popa 2021). Transovarial transmission can be through *Salmonella* colonization in the ovaries, a site of innate immune activation (Wigley 2014). The primary understanding for studying innate immunity and its relationship to host defense is regarding TLRs as ancient and evolved pattern recognition receptors (Fitzgerald & Kagan 2020). The reproductive tract contains an innate immune system with various TLRs, and TLR21 in chicken is known to be a functional homolog of TLR9 in mammals (Rehman et al. 2021). The genetics of the chicken breed strongly influence the immune response of chicken to Salmonella challenges (Kaiser et al. 2022). Indonesian local chicken have 32 breeds (Winaya et al. 2023) that are the result of the domestication of *Red junglefowl* and an important asset for the formation of superior breeds because of their ability to adapt well (Tamzil & Indarsih 2022). This study used KUB-1 chicken, which has been designated as a superior strain through the Decree of the Minister of Agriculture of the Republic of Indonesia No. 274/kpts/SR.120/2/2014, with superior characteristics in disease resistance (Sartika & Iskandar 2019). Therefore, this study aimed to characterize the molecular of TLR21 in KUB-1 chicken and their evolutionary relationships.

MATERIALS AND METHODS

Ethics statement

The protocol for tissue sampling of the reproductive tract in KUB-1 chicken in this study was approved by the Ethics Commission of the Institut Teknologi Bandung, Indonesia, through the issuance of an Ethics Decree Number 02/KEPHP-ITB/10-2019.

Sample collection

RNA samples were taken from the ovaries of reproductive adult female KUB-1 chicken that had laid eggs at least five times—collection samples from necropsied chicken with dissecting equipment. Hen ovary samples were typically taken from a tiny yellow follicle, which was then collected in cryotubes and stored at -80 °C.

RNA extraction

KUB-1 chicken ovaries stored at -80 C were removed from the freezer and waited until defrosted. Each ovary was sliced and weighed to obtain 500 mg of tissue for RNA extraction. Total RNA was extracted using the Quick-RNA Miniprep Plus Kit (R1058, Zymo Research) according to the manufacturer's protocol. NanoPhotometer® N60/N50 Microvolume Spectroscopy (Implen) was used to measure the quality and quantity of total RNA. The product from the RNA extraction was stored at -80 °C.

Reverse transcription

Total RNA was synthesized into cDNA by reverse transcription using the SensiFAST™ cDNA Synthesis

Table 1. PCR primer sequences for three fragments of the TLR21 gene in KUB-1 chicken

Primer	Sequence	TA (°C)	Size (bp)
1st Fragment	F1: TCC CAC TGC TGT CCA CTC R1: CCA GGC AAG ACG GCA GTT	58	1136
2nd Fragment	F2: GAG CTG GAC CTG TCC TGG AA R2: CAA AGC TGT CAT AGA GGT AGC A	61	1417
3rd Fragment	F3: AGT GCT GCT GCT GCT GGT R3: GAA ACT CTG TTC TGG GGA AGA	58	1343

Kit (Bioline®) according to the manufacturer's protocol. PCR was carried out with a Thermal Cycler T100™ machine (Bio-Rad). Reverse transcription conditions were set at 25 °C for 10 minutes for primer annealing, 42 °C for 15 minutes for reverse transcription, 85 °C for 5 minutes for inactivation, and 4 °C for hold. The cDNA was stored at -20 °C.

PCR amplification *TLR21* gene and sequencing

The PCR method used three pairs of primers designed based on the *Gallus gallus TLR21* sequence (NM_001030558.3). The primary design used Primer3 Plus (https://www.bioinformatics.nl/cgi-bin/primer 3plus/primer3plus.cgi) and Oligocalculator (http://biotools.nubic.northwestern.edu/OligoCalc.html). Primers were synthesized by Macrogen (Korea) with specific sequences and annealing temperatures in PCR (Table 1). PCR was run for 35 cycles with setting conditions of 95 °C for 15 seconds, 58 °C/ 61 °C for 15 seconds, and 72 °C for 10 seconds. The PCR reaction mixture used a MyTaq HS Red Mix master mix (Bioline®) with a protocol according to the manufacturer's instructions. Sequencing in this study was carried out by sending 30 µl amplicons for each KUB-1 chicken TLR21 gene fragment to Macrogen (Korea), along with 10 µl of F-primers and 10 µl of R-primers for each fragment, until Macrogen sent nucleic acid data from each fragment.

Visualization of *TLR21* fragments of KUB-1 chicken

Amplicons of the TLR21 gene were electrophoresed on 2% agarose gel at 100 volts for 35 minutes and documented by Syngene InGenius³ (Synoptic Ltd). The electropherogram of the TLR21 gene of KUB-1 chicken was read based on a 1 Kb DNA Ladder (Geneaid®).

Bioinformatics analysis

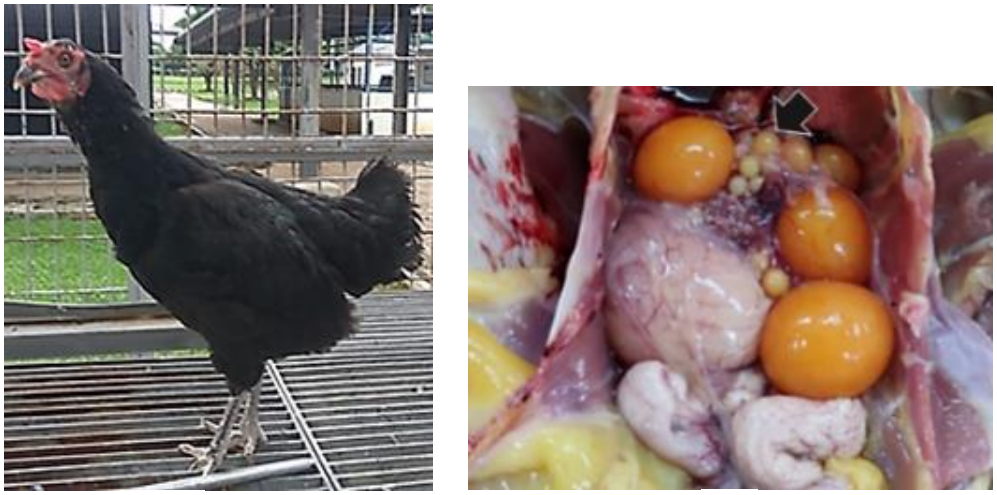
After obtaining three fragments of the TLR21 gene, the nucleic acid sequence data were processed by a bioinformatics device, trimming the nucleic acid sequence of each TLR21 fragment by Chromas version 2.6.6 (https://technelysium.com.au/wp/). BioEdit made nucleic acid sequence consensus for each TLR21 fragment—assembly for nucleic acid sequences from three KUB-1 chicken TLR21 gene fragments by Geneious Prime 2020. Furthermore, the homology of the KUB-1 chicken TLR21 nucleic acid sequence was analyzed for identity by BLAST (https://blast.ncbi.nlm.nih.gov/Blast .cgi). The orf-finder obtained the ORF area (https://www.ncbi.nlm.nih.gov/orffinder). Then, the TLR21 amino acid sequences were obtained using the ExPASy Translate tool (https://www.expasy.org/). Prediction of KUB-1 chicken TLR21 protein domain structure was made by ScanProsite (https://prosite.expasy.org/scanprosite). TLR21 amino acid sequences of chicken KUB-1 were reassigned through Multiple sequences alignment (MSA) with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The characterization of the KUB-1 chicken TLR21 included analysis to determine the amino acid sequence and the characteristics of LRRs, a TM region, TIR, hydrophobicity, solubility, molecular weight (MW), and isoelectric point (pI) of the deduced TLR21 protein. *Neighbor-Joining* constructed the phylogenetic tree with 1000x bootstrap at MEGA7.

RESULTS AND DISCUSSION

In this study, the molecular characterization of the KUB-1 chicken TLR21 gene was obtained based on the analysis of the nucleic acid sequence of cDNA TLR21 in the ovary of KUB-1 chicken and based on the amino acid sequence of the deduced protein. The evolutionary relationship of KUB-1 chicken TLR21 protein was obtained from phylogenetic analysis.

Molecular characteristic of TLR21 of KUB-1 chicken

The KUB-1 chicken used in this study was female and reproductively mature, with the character determined by the Indonesian Ministry of Agriculture based on the Decree of the Minister of Agriculture of the Republic of Indonesia No. 274/kpts/SR.120/2/2014



A B

Figure 1. Female KUB-1 Indonesian local chicken has primarily black feathers, a yellow to black beak, gray to black legs, an oval head shape, a single comb, and pea-shaped (A), the ovaries in the visceral abdomen of KUB-1 chicken, the SYF (marked with a black arrow) for RNA extraction (B)

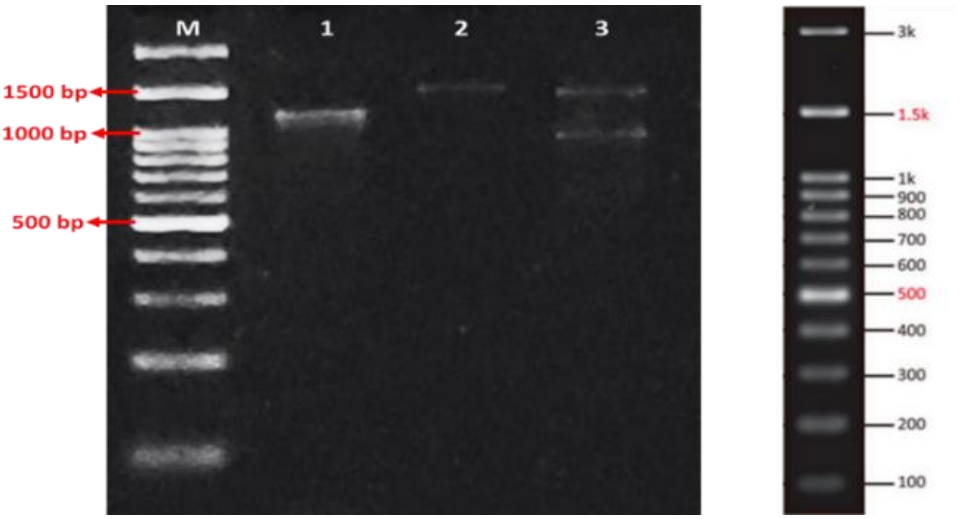


Figure 2. Electropherogram of three fragments in *TLR21* gene of KUB-1 chicken. Electrophoresis was carried out in 2% agarose gel. M= Marker (DNA Ladder 100 bp), 1= 1st fragment (1136 bp), 2= 2nd fragment (1417 bp), and 3= 3rd fragment (1343 bp)

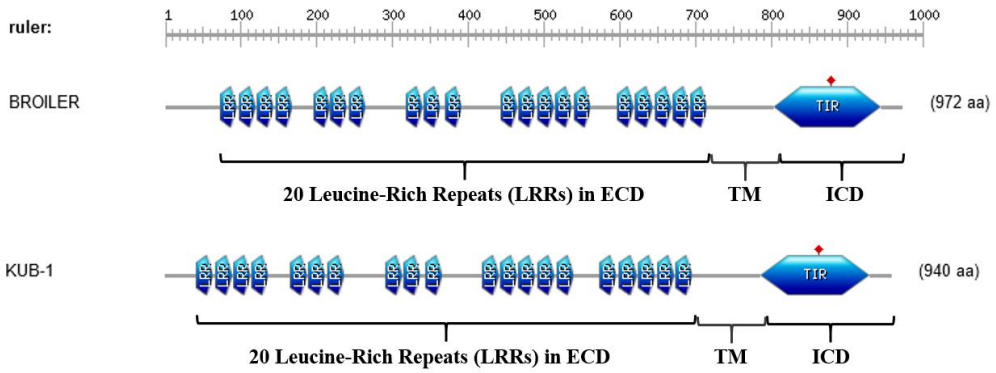


Figure 3. Characteristics of the chicken TLR21 protein profile**.** The deduced KUB-1 chicken TLR21 protein is composed of 940 amino acids. It has an extracellular domain profile containing 20 LRRs and one TIR with an active site (red diamond) in the intracellular domain, as well as broiler chicken TLR21, which is composed of 970 amino acids

(Figure 1A). The total RNA was obtained from the ovaries of KUB-1 chicken with the criteria for small and yellow ovaries (Figure 1B). Selection of ovaries with small yellow follicles (SYF) used for RNA extraction, as reported by Chen et al. (2021) that the total number of differentially expressed genes from SYF was much greater than large white follicles (LWF) and large yellow follicles (LYF). Nie et al. (2022) reported that in the ovary, there are several pre-hierarchical follicles and one hierarchical follicle at each developmental stage, and the color of the follicles gradually changes from white to yellow.

Furthermore, the measurement of RNA concentration with a nanodrop spectrophotometer obtained total RNA quality that complies with the requirements for cDNA synthesis through reverse transcription. The PCR amplification obtained three amplicons from the first, second, and third fragments in the KUB-1 chicken TLR21 gene. The electropherogram (Figure 2) showed that the length of the first fragment was 1136 bp, the second was 1417 bp, and the third was 1343 bp. A single band was obtained at the first and second fragments of the KUB-1 chicken TLR21 gene, while a double band was in the third fragment. However, the amplicons in the upper position matched the third primer target (1343 bp). In primer design, sequences with active binding sites can lead to the appearance of double bands; this is consistent with the results of ScanProsite from KUB-1 chicken TLR21, that in the third fragment, there is a TIR domain containing the active site at the residue of 847. The upper band in lane 3 will continue the sequencing process. Assembling three fragments of the KUB-1 chicken TLR21 gene, a nucleic acid sequence of 3504 bp was obtained.

Subsequently, analysis for the open reading frame (ORF) region (https://www.ncbi.nlm.nih.gov/orffinder) showed that the 3504 bp nucleic acid sequence in KUB-1 chicken TLR21 gene contained 2823 bp ORF encoded 940 aa, while in broiler chicken TLR21 (NM_001030558.3) contained 2919 bp ORF that encoding 972 aa. The prediction of protein domain structure using ScanProsite Expasy (https://prosite.expasy.org/scanprosite/) showed that in the KUB-1 chicken TLR21 and broiler chicken TLR21 (NP_001025729.3), both had extracellular domain containing 20 LRRs—illustration for the domain profile of chicken TLR21 protein as shown in Figure 3.

From the ScanProsite, the location and sequence of amino acids in each LRR and TIR can be known, but the information on the TM region cannot be obtained. The results of the analysis of the TM region of KUB-1 chicken TLR21 protein based on HMMTOP (http:// www.enzyme.hu/hmmtop/html/submit.html) found that the number of TM was only one, the length was 23 aa (LGLYLFAGTAPAVLLLLVVPVVY), and the location of the transmembrane helix sequence was in residue 721-743. The transmembrane Prediction server (https://tmdas.bioinfo.se/DAS/index.html) for the Dense Alignment Surface (DAS) curve of KUB-1 chicken TLR21 (Figure 4) illustrates the location of the TM region of this protein. Based on the results of ScanProsite on 940 aa of KUB-1 chicken TLR21 and the result of the analysis of the TM region, the amino acid sequences at LRR1–LRR20 in the ECD, TM, and TIR with the binding site in the ICD can be seen in Table 2.

It is known from Table 2 that each LRR of KUB-1 chicken TLR21 consists of 22-24 aa, where LRR17 contains 24 aa, while the other LRR consists of 22 aa. This result is consistent with the previous study (Wang et al. 2016) that each LRR module has 12-25 aa. The LRR structure with a conserved xLxxLxxLxLxxNxLxxLPxxxFx motif has a hydrophobic core, asparagine network, and variable region as reported by Manavalan et al. (2011) and illustrated as shown in Figure 5.

Each LRR of KUB-1 chicken TLR21 (Table 2) was observed to have an N (asparagine) residue, as reported by Wang et al. (2016) that among the LRR modules, there is a conserved asparagine which plays a vital role in maintaining the shape of the ECD by forming a continuous hydrogen bond network and forming the asparagine ladder. Asparagine can be replaced by other amino acids capable of donating hydrogens, such as threonine, serine, and cysteine. Furthermore, asparagine contained in each LRR of KUB-1 chicken TLR21 has an intact asparagine ladder on the concave surface of the adjacent LRR module to stabilize the overall shape of the ECD responsible for ligand recognition. The ECD of single-domain TLRs with intact asparagine ladders allows for binding to nucleic acids or hydrophilic proteins.

Furthermore, Matsushima et al. (2007) and Batkhishig et al. (2020) reviewed that LRR has a highly conserved segment (LRRh) and a variable segment (LLRv), where the LLRh motif is LxxLxLxxNxL or LxxLxLxxNxxL or LxxLxLxxCxxL. The LRRh motif at KUB-1 chicken TLR21 was found in LRR2 (QLHTLDLTYNLLETLSPGAFNG). In LRR3, 14-17 KUB-1 chicken TLR21 also has the LRRh motif. These results are similar to the previous studies (Li et al. 2018) that found that LRR2, LRR3, and LRRs14-17 have an LRR Typ. Meanwhile, Wu et al. (2018) reported that LRRs 14-18 had relatively higher conserved scores than other LRR motifs, implying that they have a role in protein-protein interfaces for dimerization or binding to other members of the TLR family. Matsushima et al. (2007) reported that other hydrophobic amino acids can replace the conserved L residue. Meanwhile, LRR4 (NLSSLQVQHNPLSTVSPSALLP) and LRR10 (KLEVLTVEHNLLKKLPSCLGAQ) in the L residue are replaced by V (Val), in which the "L" residue is Leu, Ile, Val, or Phe. Four L residues at amino acids at position.

Table 2. Amino acid positions and sequences in the domain of KUB-1 chicken TLR21

Domain	LRR	Aa position	Aa Sequences
ECD	1	42 – 63	HAIALNLSYNKMRCLQPSAFAH
	2	66 – 87	QLHTLDLTYNLLETLSPGAFNG
	3	90 – 111	VLVVLDLSHNKLTTLAEGVFNS
	4	114 – 135	NLSSLQVQHNPLSTVSPSALLP
	5	164 – 185	QLELLDLCENNLTTLGPGPPLP
	6	187 – 208	SLLTLQLCNNSLRELAGGSPEM
	7	211 – 232	HVKILDLSYNSISQAEVFTQLH
	8	286 – 307	ALRRLQLQRNGLKVLHCNALQL
	9	310 – 331	VLRELDLSWNRLQHVGCAGRLL
	10	338 – 359	KLEVLTVEHNLLKKLPSCLGAQ
	11	411 – 432	NLTELRLDNNLLTDLYHNSFID
	12	435 – 456	RLRTLNLRNNRVSVLFSGVFQG
	13	459 – 480	ELQTLDLGGNNLRHLTAQSLQG
	14	483 – 504	KLRRLYLDRNRLLEVSSTVFAP
	15	508 – 529	TLGVLDLRANNLQYISQWLRKP
	16	564 – 585	RLQQLSLSQNMLRSIPPDVFED
	17	588 – 611	QLRSLALadSSNGLHDLPDGIFRN
	18	614 – 635	NLRFLDLENAGLHSLTLEVFGN
	19	638 – 659	RLQVLHLARNELKTFNDSVASR
	20	662 – 683	SLRYLDLRKCPLSCTCDNMWLQ
TM		721 – 743	LGLYLFAGTAPAVLLLLVVPVVY
ICD	TIR	772 – 912	YLYDSFVSYNSADESWVLQKLVP
			ELEHGAFRLCLHHRDFQPGRSIIDN
			IVDAVYNSRKTVCVXSRSYLRSEW
			CSLEVQLAsYRLLDERRDILVLVLL
			EDVGDAELSAYHRMRRVLLRRTY
			LRWPLDPAAQPLFWARLKRAL

Conserved sequences in the TIR domain are marked in the box. ECD= extracellular domain, LRR= leucine-rich repeat, TM= transmembrane, ICD= intracellular domain, TIR= Toll-IL-1 receptor

positions 1, 4, 6, and 11 participate in the hydrophobic core in the LRR arc. The LRRv motif in KUB-1 chicken TLR21 was thought to be present in LRR1 (HAIALNLSYNKMRCLQPSAFAH) and LRR20 (SLRYLDLRKCPLSCTCDNMWLQ) consistent with Matsushima et al. (2007) that the first LRR in the N-terminus (LRR1) is often irregular, for example, (L/x)xx(L/A)xCxx(L/R)xLxxVPxxIPxx, and most LRR structures have closures containing two or four cysteine residues to protect the hydrophobic core from the first LRR at the N-terminus (LRRNT) or the last LRR at the C-terminus (LRRCT).

The TIR of KUB-1 chicken TLR21 is located at residues 772-912, with the active site at the amino acid position 847 (marked by E in bold in the TIR sequence). The number of amino acids that comprise the TIR in KUB-1 chicken TLR21 consists of 141 aa. This result is similar to the TIR domain structure in chicken TLR21 predicted by Wu et al. (2018) that there is about 125-200 aa in the TIR domain, which is required for protein-protein interactions and the position of the 778-943 residue forms a highly conserved structure in the cytoplasmic region of chicken TLR21. The results of this study, in the TIR domain of KUB-1 chicken TLR21,

obtained three conserved sequences: YDSFVSYN, LCLHHRDFQPGR, and LFWAR, whose sequences were marked in boxes. A previous Li et al. (2018) study reported that the conserved sequences in boxes 1 and 2 mediate receptor molecules in signal transduction pathways. In contrast, the function of the sequences in box 3 is to control the subcellular location of these receptors. Meanwhile, Chuang et al. (2020) also reported three conserved sequences in the TIR domain of the TLR, in which TIR-conserved sequences for mammalian TLR signaling were also conserved in the TLR21.

Based on previous data that KUB-1 chicken TLR21 protein had a hydrophobic core region in each LRR, the hydrophobicity analysis (https://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi?rm=misc1) and solubility analysis (https://protein-sol.manchester.ac.uk/) were carried out by the Kyle-Dolittle plot on the hydropathy curve of KUB-1 chicken TLR21 protein (Figure 6A). The characteristics of the ECD and the TM region of KUB-1 chicken TLR21 tend to be hydrophobic, while the TIR domain tends to be hydrophilic. The results of the solubility scale for KUB-1 chicken TLR21 protein was 0.208 with a solubility value <0.45 (Figure 6B) that was predicted to be insoluble in E. coli (assessed to have a low probability of being expressed in E. coli). Hebditch et al. (2017) described this result as the solubility value scale (QuerySol), the predicted solubility. The population means for the experimental dataset (PopAvrSol) was 0.45. Therefore, a scaled solubility value greater than 0.45 was predicted to have a higher solubility than the mean from the experimental solubility dataset of *E.coli*-soluble protein, and any protein with a scale solubility lower than 0.45 was expected to be less soluble. In addition, the analysis of molecular weight (MW) and isoelectric point (pI) (https://web.expasy.org/ compute_pi/) showed that KUB-1 chicken TLR21 protein has 9.09 pI and 106,947.22 Da of MW.

Furthermore, the multiple sequence alignments (MSA) for amino acids sequences using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) that retrieved the TLR sequences from the database in the Protein Database (PDB) obtained the homology of the tertiary structure of KUB-1 chicken TLR21 protein vs. other vertebrate TLRs, and the top three results are shown at

Table 3. BLAST results of KUB-1 chicken TLR21 protein sequences aligned with sequences in the Protein Database (PDB)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
TLR7 [*Homo sapiens*]	*Homo sapiens*	162	162	67%	4e-40	28.02%	1049	7CYN_A
TLR13-ssRNA13 complex [*Mus musculus*]	*Mus musculus*	155	155	75%	1e-38	29.61%	709	4Z0C_A
TLR5 fitted into an electron microscopy [*Homo sapiens*]	*Homo sapiens*	137	191	64%	1e-32	26.99%	844	3J0A_A

Table 4. BLAST result of KUB-1 chicken TLR21 protein sequence

Description	Query Cover	E value	Per. Ident	Acc. Len	Accession
TLR21 *Gallus gallus* breed Beijing White 939	97%	0.0	99.89%	972	AFD61602.1
TLR21 *Gallus gallus* (broiler >< layer, bGalGal1)	97%	0.0	99.89%	967	XP_040562958.1
TLR21 *Gallus gallus* (broiler.GRCg7b)	97%	0.0	99.89%	972	NP_001025729.3
TLR21 *Gallus gallus* breed White Leghorn	97%	0.0	99.78%	972	AFD61603.1
TLR21 *Gallus gallus* breed White-Feather Silky	97%	0.0	99.45%	972	AFD61606.1
TLR21 *Gallus gallus* breed Nongda No.3	97%	0.0	99.56%	972	AFD61605.1
TLR21 *Anas platyrhynchos*	97%	0.0	75.90%	976	AUO17544.1
TLR21 *Anser cygnoides*	97%	0.0	76.89%	976	AMB20882.1
TLR21 *Danio rerio*	97%	0.0	43.66%	989	CAQ13807.1
TLR21 *Cyprinus carpio*	98%	0.0	43.96%	964	AVX48323.1

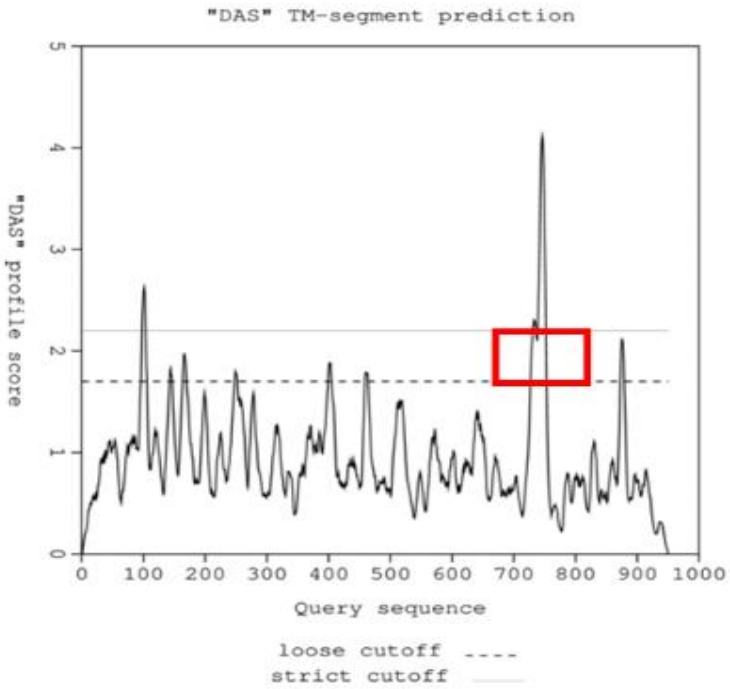


Figure 4. DAS curve of KUB-1 chicken TLR21 protein with a red box in the curve indicates the TM region. This curve is obtained by pairwise comparison of the proteins in the test set in an "each against the rest" fashion. Two cutoffs are on the plots: a "strict" one at 2.2 DAS score and a "loose" one at 1.7. The hit at 2.2 is informative in terms of the number of matching segments, while a hit at 1.7 gives the actual location of the TM segment

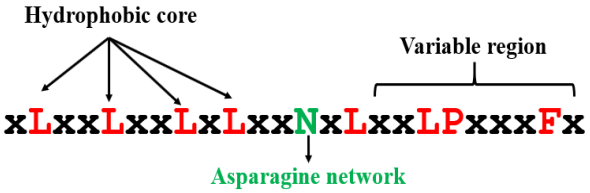


Figure 5. Consensus for LRR. Amino acid residues form a hydrophobic core, asparagine ladder, and variable regions. L, P, and F for Leucine, Proline, and Phenylalanine in red = hydrophobic, N for Asparagine in green = hydroxyl/amine/base, and x for any amino acid

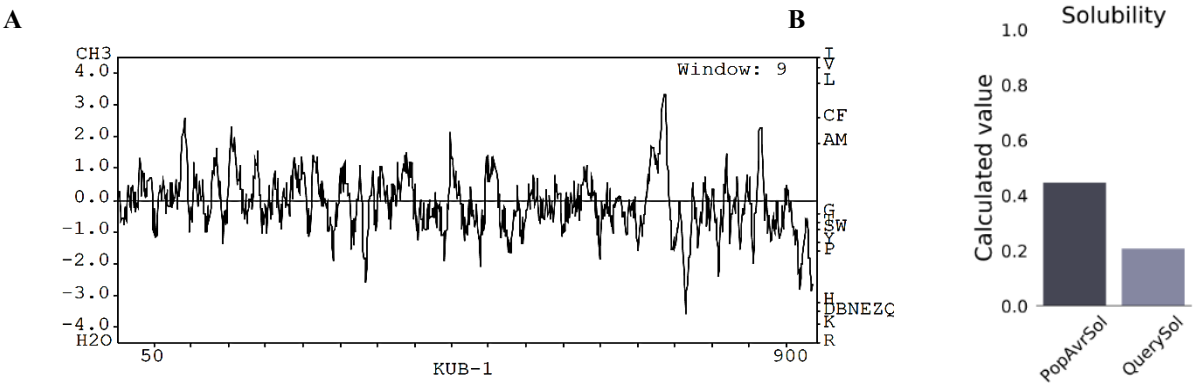


Figure 6. Predicted hydrophobicity and solubility of KUB-1 chicken TLR21 protein. KUB-1 chicken TLR21 protein tends to be hydrophobic (A); the predicted solubility scale for KUB-1 chicken TLR21 protein was 0.208, with a solubility value <0.45 (B)

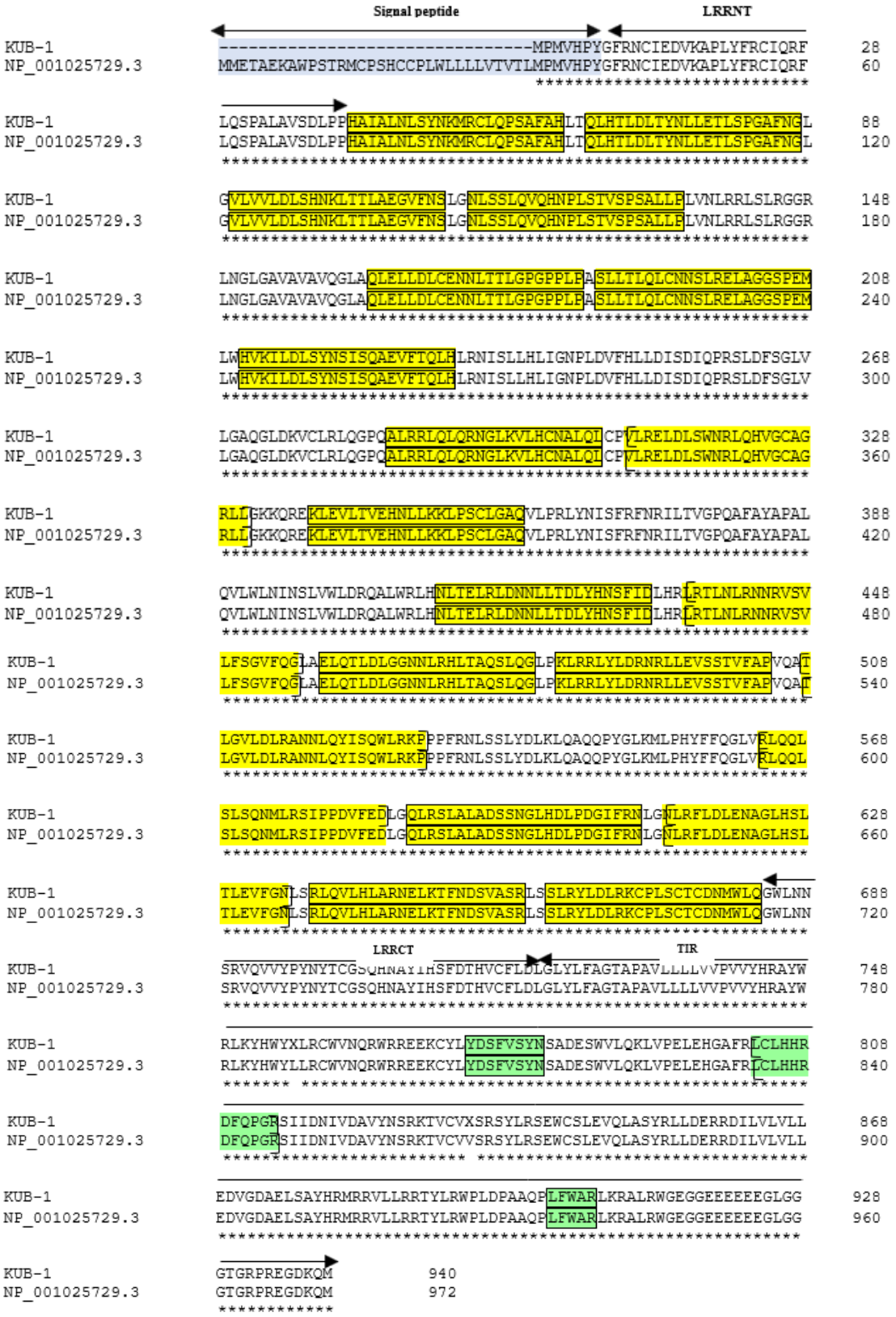


Figure 7. MSA Clustal Omega of KUB-1 chicken TLR21 to broiler TLR21 (NP_001025729.3). The LRR1–LRR20 is highlighted in yellow, and three conserved sequences in the TIR domain are highlighted in green

Table 3. The result showed that the deduced protein of KUB-1 chicken TLR21 had a homology of ~30% against *M. Musculus* TLR13, *H. sapiens* TLR7, and *H. sapiens* TLR5. Wang et al. (2016) reported that the chicken TLR21 has a single-domain architecture which is also shared by TLR5 (Family 5), TLR7 (Family 7), and TLR13 (Family 11). In addition, the results of MSA Clustal Omega (Figure 7) showed that in the middle of the KUB-1 chicken LRR sequence, there was a region containing 51 residues between LRR10 and LRR11. Matsushima et al. (2007) reported that the TLR in Family 7 includes a region containing 58-73 residues in the middle part of the LRR domain, where the middle has a vital role in the structure and function of the TLR. Regarding the homology to TLR13, Wu et al. (2018) reported that the number of LRR motifs in *G. gallus* TLR21 had similarities with *M. musculus* TLR13 and *M. musculus* TLR9, where the individual LRR consisted of about 22-33 residues, except for the LRRCT and LRRNT sequences in the ECD of chicken TLR21. Then, structural comparison of the ECD and the TIR domain in the TLR was reported by Wu et al. (2018) that in the chicken TLR21, there is no Z-loop between the LRR14 and LRR15 modules in the center of the chicken TLR21 ECD, which is very different from the crystal structure of the human TLR9 ECD (PDB code: 3wpc), but similar to the TLR13 ECD (PDB code: 4z0c). Therefore, although chicken TLR21 is a mammalian TLR9 homology, the structural biology of the chicken TLR21 ECD structure does not have a Z-loop as found in mice TLR9. Subsequently, the homology of the amino acids sequence of KUB-1 chicken TLR21 against other TLR21 based on the BLAST result showed that the deduced protein of KUB-1 chicken TLR21 had homology of about 43%, 76%, and 99% against fish TLR21, non-chicken poultry TLR21, and other breed chicken TLR21 respectively, as shown in Table 4.

Phylogenetic analysis of TLR21 protein of KUB-1 chicken

To determine the relationship between KUB-1 chicken TLR21 protein and other vertebrate TLRs, the amino acid sequence data from Table 3 and Table 4 were used in the phylogenetic analysis. A phylogram (Figure 8) was constructed for the KUB-1 chicken TLR21 amino acid sequence for evolutionary kinship with the vertebrate TLRs. The result shows that *Gallus galus*_KUB-1 TLR21 and *Mus musculus* TLR13 are in one cluster. This result is consistent with the phylogenetic analysis in a previous study that TLR21 and TLR13 were classified in Family 11 (Wang et al. 2016) due to both of them having an intact asparagine ladder in single domain architecture at the ECD of their LRRs.

Subsequently, to determine the evolutionary kinship of KUB-1 chicken TLR21 against TLR21 from duck, goose, fish, and other breeds of chicken, the data in Table 4 were used in the phylogenetic analysis, and the phylogram (Figure 9) shows that the KUB-1 chicken TLR21 is an ortholog of the TLR21 fish (*Danio rerio* and *Cyprinus carpio*). As reported in a previous study by Lai et al. (2019), this result shows that chicken TLR21 is the ortholog of TLR21 in fish and amphibians. Meanwhile, Rehman et al. (2021) reported that the paralogue in the chicken TLR comes from gene duplication, and the chicken TLR only has two paralogs. Due to TLR21 being a non-mammal TLR type, this phylogenetic analysis was not compared to the mammalian TLR21 reported by Priyathilaka et al. (2014). The phylogenetic analysis showed that TLR21 in this study still conserved evolutionarily, consistent with Rehman et al. (2021), who found that TLRs in chicken have exhibited conserved traits during evolution.

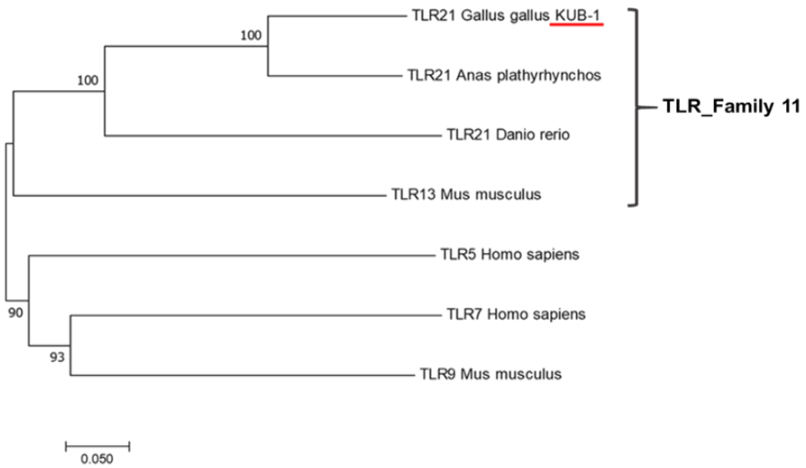


Figure 8. Phylogram of KUB-1 chicken TLR21 against other vertebrate TLRs constructed using the Neighbor-Joining algorithm with 1000x bootstrapping of the TLR21 amino acid sequence that has been aligned with ClustalW on MEGA7 shows that TLR21 in birds and fish are grouped in one cluster with *Mus musculus* TLR13, while *Mus musculus* TLR9, *Homo sapiens* TLR5 and *Homo sapiens* TLR7 were in a different cluster

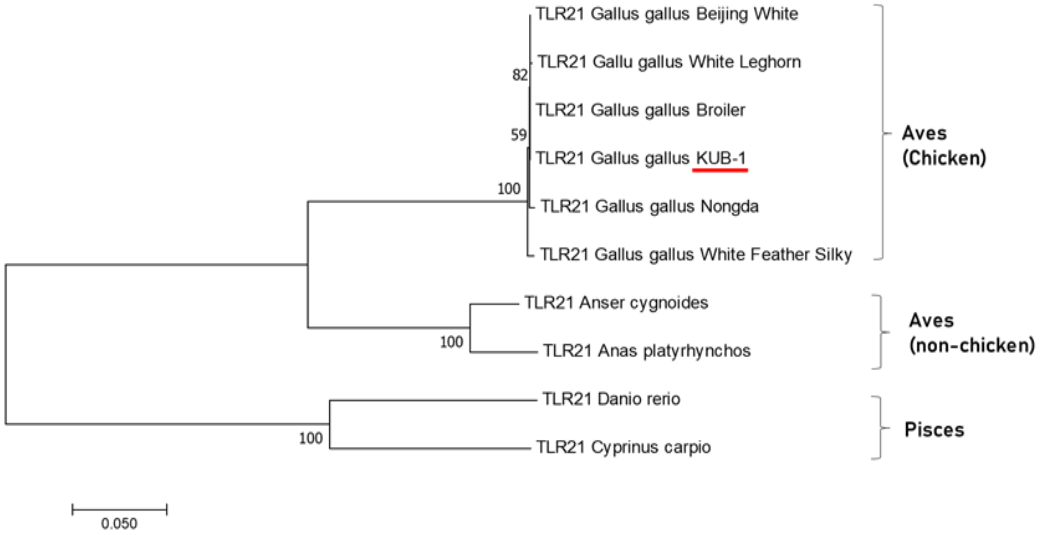


Figure 9. Phylogram of KUB-1 chicken TLR21 against other TLR21. The tree constructed using the Neighbor-Joining algorithm with 1000x bootstrapping on the amino acid sequences of TLR21 that have been aligned with ClustalW on MEGA 7 shows that TLR21 from Aves is grouped in one cluster, which is divided into two sub-clusters: chicken TLR21 and non-chicken (duck and goose) TLR21. In contrast, Pisces TLR21 is in a different cluster from Aves TLR21. The phylogenetic tree showed that *Gallus gallus* TLR21 formed a paraphyletic relationship with TLR21 in *Anser cygnoides* and *Anas platyrhynchos*

CONCLUSION

The molecular characteristic of KUB-1 cDNA TLR21 has nucleotides 3504 bp, which include a 2823 bp ORF, which encodes putative proteins of 940 aa. The deduced protein consisted of 720 aa, 23 aa, and 141 aa in ECD, TM, and ICD, respectively. Evolutionarily, KUB-1 chickens' TLR21 protein, which belongs to the TLR11 family, has differentiated from fish and formed a paraphyletic relationship with ducks and geese.

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