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Original Article

Identification of Avian Toll-Like Receptor 3 and 7 and Analysis of Gene Variation Sites

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ABSTRACT

Toll-like receptors 3 and 7 (TLR3 and 7) mediate immune responses through the recognition of viral single-stranded RNA and double-stranded RNA and therefore play important roles in host defense. Differences in TLR3 or 7 may affect host resistance to RNA viral infection. To illuminate these differences, the partial coding sequence (CDS) of TLR3 and 7 genes were cloned and amplified from the *Phasianus colchicus* and *Numida meleagris*, total 64 avian species of TLR3 and 7 sequences were later analyzed. Based on the results, 315 non-synonymous mutation sites and 202 synonymous mutation sites were also observed in the avian TLR3, and 227 non-synonymous mutation sites and 174 synonymous mutation sites were observed in the avian TLR7. Among these sites, 44 and 45 sites were observed in functional regions of TLR3 and 7, used common variation of amino acids in most avian species. A number of these different sites appeared to affect the recognition and were also visualized. H59Y, E60K, G64R, E93K, L112S, K117E, N118K, R120H, V123M, L163F, R443Q, R459K, E460D, C485H, and F511L for TLR3, and I432V, M437V, and T732S for TLR7 were considered. It is possible that these sites bind to ligands and play crucial roles in viral recognition. These data indicated that the positive selection has occurred in the avian TLR3 and 7 genes.

INTRODUCTION

Toll-like receptors (TLRs) are the most-characterized receptors among the pattern recognition receptors (PRRs). PRRs are recognized as a diverse range of pathogen associated molecular patterns (PAMPs) and play a critical role in antimicrobial host defense (Medzhitov, 2001). Toll-like receptors are critical proteins linking innate and acquired immunity (Akira, 2001). TLRs are evolutionarily conserved with homologs present in insects, fish, mammals, and birds (Kimbrell & Beutler, 2001; Roach *et al.*, 2005; Satake & Sasaki, 2010; Valanne *et al.*, 2011). Ranges of TLR genes have been identified in avian species (Boyd *et al.*, 2001; Fukui *et al.*, 2001; Iqbal *et al.*, 2005; Philbin *et al.*, 2005; Yilmaz *et al.*, 2005; Brownlie & Allan, 2011; Keestra *et al.*, 2013). All avian TLR family have 10 members that include TLR1 type 1 and type 2, TLR2 type 1 and type 2, TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21. However, not all birds have exact 10 TLRs. Several species have duplicated TLR7, others not functional TLR5 (Brownlie & Allan, 2011; Velova *et al.*, 2018). TLR1, TLR2, TLR4, and TLR5 have recognized bacterial components, such as peptidoglycans (PGN), lipopolysaccharides (LPS), cell wall lipids, and flagellum (Brownlie & Allan, 2011). TLR15 has recognized yeast-derived components (Boyd *et al.*, 2012). TLR21 has recognized microbial DNA and is homologous with fish (Keestra *et al.*, 2010). The recognition of viral RNA depends on TLR3 and TLR7, which recognize viral double-stranded RNA and single-stranded RNA (Alexopoulou *et al.*, 2001; Heil *et al.*, 2004).

Due to the important roles in resistance to pathogen invasion, TLR genes have been conserved throughout evolution. Mutations in TLRs may have a profound influence on the host's response to pathogens and are also associated with resistance to and susceptibility to diseases (Chen *et al.*, 2009; Hawn *et al.*, 2009; Al-Qahtani *et al.*, 2012; Goyal *et al.*, 2012; O'Dwyer *et al.*, 2013). There are many studies researching mutations in human TLRs (Misch & Hawn, 2008; Mukherjee *et al.*, 2019). Several studies have focused on avian TLR gene mutations and polymorphisms (Alcaide & Edwards, 2011; Ruan *et al.*, 2012; Ruan *et al.*, 2015; Swiderska *et al.*, 2018; Velova *et al.*, 2018). In the present study, we explored different genetic patterns of *TLR3* and *7* in the *Phasianus colchicus*, *Numida meleagris*, and other 62 avian species. The results were helpful to understand the genetic evolution of avian *TLR3* and *7*.

MATERIALS AND METHODS

Sources of avian breeds

The *Numida meleagris* and the *Phasianus colchicus* used in this study were obtained from Beijing Shahe breeder. We arranged four repeats of individuals. All procedures were approved by the Animal Care and Use Committee of Beijing University of Agriculture (Beijing, China).

Molecular cloning of TLR3 and 7 and sequence accession number

The total RNA was obtained from spleens using *TRIzol* (Invitrogen, USA). The total RNA, a random primer, dNTPs, and *M-MLV* reverse transcriptase (Promega, USA) were used for cDNA synthesis. PCR was performed to amplify the target gene using three pairs of specific primers for *TLR3* and four pairs of specific primers for *TLR7* (Table1). PCR reactions were performed with *pfu* polymerase (Promega, USA). The 25 µl PCR reaction contained 50 pmol of each forward and reverse primers, 2 µl template cDNA, 200 µM each of deoxynucleotide triphosphate mixture and 2.5 U *Pfu* DNA polymerase (Promega) in 1× *Pfu* DNA polymerase buffer. Amplification conditions were as follows: initial denaturation at 94°C for 2 min, 35 cycles at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 3 min, followed by a final extension at 72°C for 10 min. PCR amplicons were verified by 1% agarose gel electrophoresis, then ligated into a *pEASY-Blunt* simple cloning vector (TransGen, Beijing, China). Recombinant plasmids were characterized by PCR using gene specific and vector primer pairs. Recombinant plasmids with avian *TLR3* and *7* were sequenced from both ends by Sangon Biotech Co., Ltd. Sequences for *TLR3* and *7* for *Numida meleagris* and the *Phasianus colchicus* were deposited in the GenBank database under accession number MG604328-MG604332. Other sequences of TLR3 and 7 of 62 avian species were acquired from GenBank, the accession numbers are shown in Table 2.

Analysis of variation sites

Nucleotide sequences for avian TLR3 and TLR7 were aligned in MegAlign by the ClustalW method (DNAstar version 8.1.3). The nucleotide homology was showed in a report of MegAlign. The functional regions were detected with the analysis tools provided at the website (http://smart.embl-heidelberg.de and http://split.pmfst.hr). The relative frequency of non-synonymous (dN) and synonymous (dS) substitutions was calculated and constructed using the MEGA7 software (version 7) (Kumar, *et al.*, 2016). Crystal structures and non-synonymous sites in the avian TLR3 and 7 were visualized by PDB (Protein Data Bank, ID:1ZIW and 5GMF) models and PyMOL software (version 2.3, DeLano Scientific LLC).

RESULTS AND DISCUSSION

The full-length open reading frame (ORF) for avian *TLR3* was 3036 nucleotides that encoded 1011 amino acids. The ORF for avian *TLR7* was 3180 nucleotides and encoded 1059 amino acids. Partial nucleotide sequences were also analyzed for homology. The nucleotide sequence alignment showed that *Phasianus colchicus* and *Numida meleagris* share 93.6%-95% homology with chicken *TLR3* and *7*. The homology between *Numida meleagris* and the *Phasianus colchicus* was 92.8%-94.1% for *TLR3* and *7*.

The extracellular domain of the TLR, especially the leucine-rich repeat domain (LRR), is a region for recognizing pathogens (Jin and Lee, 2008; Werling, *et al.*, 2009). Cytoplasmic domains of the TLR, especially the Toll/interleukin-1 receptor domain (TIR), is a region for signal transduction (Verstak, *et al.*, 2009). Three hundred and fifteen non-synonymous mutation sites and 202 synonymous mutation sites were observed in the avian *TLR3*, including 60 sites for *Numida meleagris* and 44 sites for the *Phasianus colchicus*. Sixty-two variable sites were located in the extracellular domain, including 34 sites in LRR regions. Eighteen variable sites were also located in the cytoplasmic domain, including 9 sites in the TIR region. Two hundred and twenty-seven non-synonymous mutation sites and 174 synonymous mutation sites were observed in the avian *TLR7*, including 58 sites for *Numida meleagris* and 64 sites for the *Phasianus colchicus*. Eight-two variable sites were located in the extracellular domain, including 34 sites in the LRR domain, and 8 variable sites were located in the cytoplasmic domain, including 2 sites in the TIR domain. Two sites were located in the transmembrane domain. Among these sites, 44 amino acid sites were observed in LRR and TIR region of TLR3 and used common variation of amino acids in most avian species (Table 3), including G64R, L163F, and H627Y in α-helix structure, V123M, V224R, N381S, and E383Q in β-sheet structure (Figure 1). Forty-five mutation sites were also observed in LRR and TIR region of *TLR7* and used common variation of amino acids in most avian species (Table 4), including T175N, F176L, and E341Q sites in α-helix structure, F51S, R56T, V216I, Q664E, I689V, and I739M in β-sheet structure (Figure 1). These sites located in α-helix or β-sheet may affect structure of TLR and recognition function.

Three-dimensional structures of avian TLR3 and TLR7 were helpful for further speculating on the role of these variable sites (Choe *et al.*, 2005). The sites that are located at the external and LRR domains may be more important than others (Botos, *et al.*, 2011). Moreover, it reported that two N-terminal half-sites in both dimer subunits of TLR3 are the viral dsRNA binding sites (Alexopoulou *et al.*, 2001; Leonard *et al.*, 2008; Liu *et al.*, 2008; Zhang *et al.*, 2017). In this study, H59Y, E60K, G64R, E93K, L112S, K117E, N118K, R120H, V123M, L163F, R443Q, R459K, E460D, C485H, and F511L for avian TLR3 were considered in all 51 avian species (Figure 1). The sites located at long loop of TLR7, termed 'Z-loop', may also be the viral ssRNA binding sites (Zhang *et al.*, 2016; Zhang *et al.*, 2017; Diebold *et al.*, 2004). In this study, I432V, M437V, and T732S for avian TLR7 were considered in all 49 avian species (Figure 1). These sites may bind to ligands and play crucial roles in viral recognition. Fully characterizing the functions of these sites would require a large number of experiments.

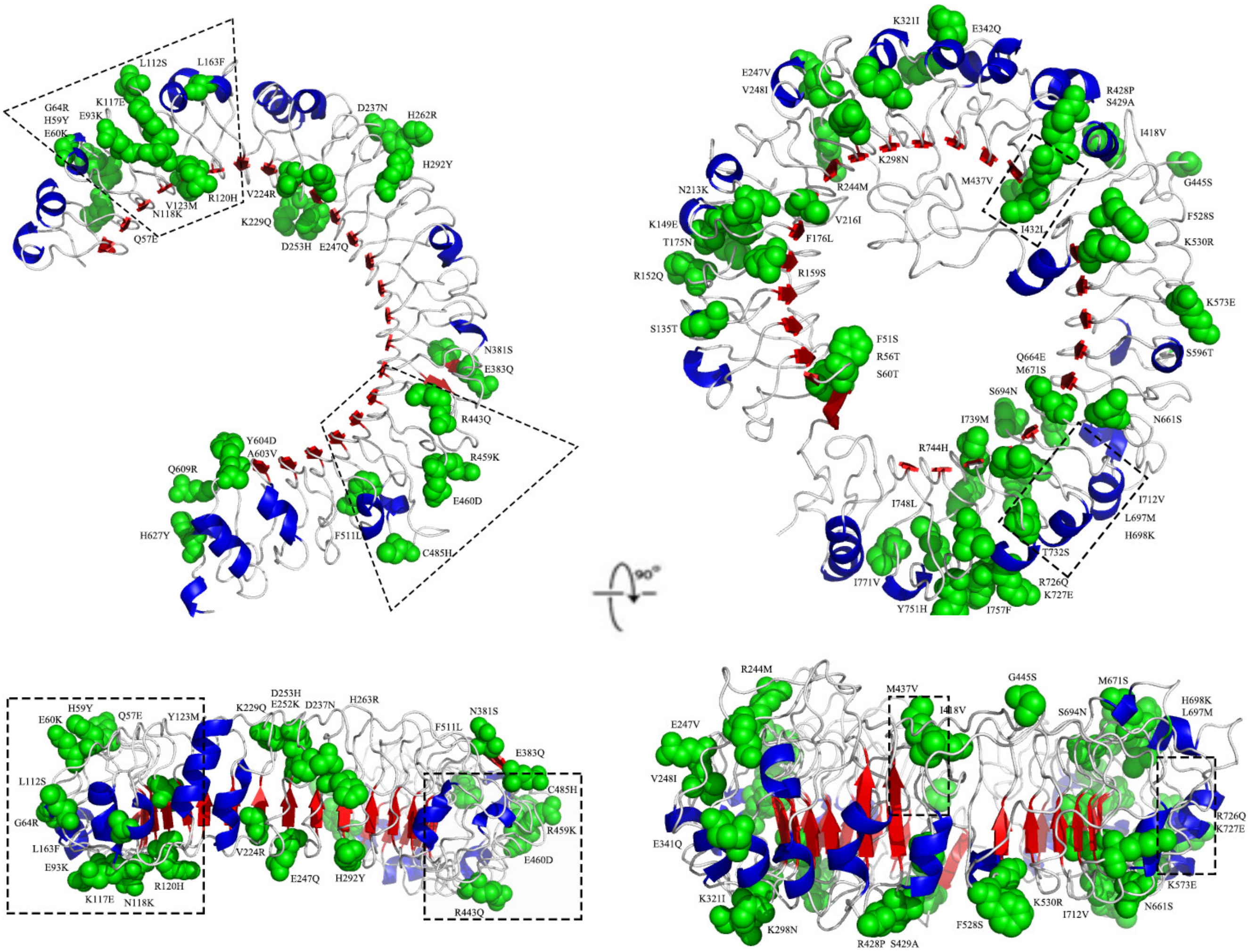

Figure 1 – Visualization of amino acids corresponding to nonsynonymous single nucleotide variations in the extracellular domain of avian *TLR3* (left) and *TLR7* (right) based on the protein structure predicted by CPHmodels 3.0. The predicted ligand binding sites were marked with dotted box.

There is mounting evidence suggesting that there are species-specific components to TLR (Werling *et al.*, 2009). Differences in avian TLR3 and TLR7 reflect the differences in geography and microbial environments (Liu *et al.*, 2006; Alcaide & Edwards, 2011). These variable TLRs recognize the same or similar pathogens and perform the same functions. Different avian TLR3 and TLR7 sequences could also affect the host's resistance to viruses. These variable sites in the extracellular LRR domain, especially the viral binding sites that have been reported, play a dramatic role in recognizing viruses. Further functional research regarding these differences may clarify the impacts of these variable sites in avian *TLR3 and 7*.

The dS/dN represents the proportion between the Ka (Synonymous mutation) and the Ks (non-synonymous mutation). This ratio determines whether there was any selective pressure on the *TLR3* and *7* genes. This finding indicates positive selection occurred in avian *TLR3* and *7* genes because the frequency of synonymous (dS)/frequency of non-synonymous (dN) (dS/dN of *TLR3* is 0.64; dS/dN of *TLR7* is 0.77). Mutations will be retained in avian *TLR3* and *7*. This property was highly conserved through gene evolution and the more important function of recognizing viruses (Liu *et al.*, 2006; Bergman *et al.*, 2010; Alcaide & Edwards, 2011).

Based on the TLR3 and 7 polymorphisms and their correlations with human and mouse susceptibility to viral infections, we propose that avian TLR3 and 7 differences may be associated with either resistance or susceptibility to avian infectious diseases (Schott *et al.*, 2007; Lee *et al.*, 2013; Piaserico *et al.*, 2015; He *et al.*, 2017). This study may be helpful to further understand the varied resistance to viral diseases that exist between different avian species.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: WR. Performed the experiments: YL QL. Analyzed the data: YL WR. Contributed reagents/materials/analysis tools: WR. Wrote the paper: WR.[1]

REFERENCES

Akira S. Toll-like receptors and innate immunity. Advances in Immunology 2001;78:1-56.

Alcaide M, Edwards SV. Molecular evolution of the toll-like receptor multigene family in birds. Molecular Biology and Evolution 2011;28:1703-15.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-8.

Al-Qahtani A, Al-Ahdal M, Abdo A, Sanai F, Al-Anazi M, Khalaf N, *et al.* Toll-like receptor 3 polymorphism and its association with hepatitis B virus infection in Saudi Arabian patients. Journal of Medical Virology 2012;84:1353-9.

Bergman IM, Rosengren JK, Edman K, Edfors I. European wild boars and domestic pigs display different polymorphic patterns in the Toll-like receptor (TLR) 1 TLR2 and TLR6 genes. Immunogenetics 2010;62:49-58.

Botos I, Segal DM, Davies DR. The structural biology of Toll-like receptors. Structure 2011;19:447-59.

Boyd AC, Peroval MY, Hammond JA, Prickett MD, Young JR, Smith AL. TLR15 is unique to avian and reptilian lineages and recognizes a yeast-derived agonist. Journal of Immunology 2012;189:4930-8.

Boyd Y, Goodchild M, Morroll S, Bumstead N. Mapping of the chicken and mouse genes for toll-like receptor 2 (TLR2) to an evolutionarily conserved chromosomal segment. Immunogenetics 2001;52:294-8.

Brownlie R, Allan B. Avian toll-like receptors. Cell Tissue Research 2011;343:121-30.

Chen Z, Ma G, Qian Q, Yao Y, Feng Y, Tang C. Toll-like receptor 8 polymorphism and coronary artery disease. Molecular Biology Reports 2009;36:1897-901.

Choe J, Kelker MS, Wilson IA. Crystal structure of human toll-like receptor 3 (TLR3) ectodomain. Science 2005;309:581-5.

Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 2004;303:1529-31.

Fukui A, Inoue N, Matsumoto M, Nomura M, Yamada K, Matsuda Y, *et al.* Molecular cloning and functional characterization of chicken toll-like receptors A single chicken toll covers multiple molecular patterns. Journal of Biological Chemistry 2001;276:47143-9.

Goyal S, Dubey PK,Tripathy K, Mahajan R, Pan S, Dixit SP, *et al.* Detection of polymorphism and sequence characterization of Toll-like receptor 7 gene of Indian goat revealing close relationship between ruminant species. Animal Biotechnology 2012;23:194-203.

Hawn T, Scholes RD, Li SS, Wang H, Yang Y, Roberts PL, *et al.* Toll-like receptor polymorphisms and susceptibility to urinary tract infections in adult women. PloS One 2009;4:e5990.

He H, Liu S, Liu PP, Li QB, Tan YX, Guo Y, *et al.* Association of Toll-like receptor 3 gene polymorphism with the severity of enterovirus 71 infection in Chinese children. Archives Virology 2017;162:1717-23.

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, *et al.* Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;303:1526-9.

Iqbal M, Philbin V J, Withanage GS, Wigley P, Beal RK, Goodchild MJ, *et al.* Identification and functional characterization of chicken toll-like receptor 5 reveals a fundamental role in the biology of infection with Salmonella enterica serovar typhimurium. Infection and Immunity 2005;73:2344-50.

Jin MS, Lee JO. Structures of the toll-like receptor family and its ligand complexes. Immunity 2008;29:182-91.

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[1] Tables 1 to 4 are presented in the pages that follow the references.

Keestra AM, Zoete MR de, Bouwman LI, Vaezirad MM, Putten JP van. Unique features of chicken Toll-like receptors. Developmental and Comparative Immunology 2013;41:316-23.

Keestra AM, Zoete MR de, Bouwman LI, Putten JP van. Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9. Journal of Immunology 2010;185:460-7.

Kimbrell DA, Beutler B. The evolution and genetics of innate immunity. Nature Reviews Genetics 2001;2:256-67.

Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis. Version 70 for Bigger Datasets. Molecular Biology and Evolution 2016;33:1870-4.

Lee SO, Brown RA and Razonable RR. Association between a functional polymorphism in Toll-like receptor 3 and chronic hepatitis C in liver transplant recipients. Transplante Infectious Diseases 2013;15:111-9.

Leonard JN, Ghirlando R, Askins J, Bell JK, Margulies DH, Davies DR and Segal DM. The TLR3 signaling complex forms by cooperative receptor dimerization. Proceedings of National Academy Science USA 2008;105:258-63.

Liu L, Botos I, Wang Y, Leonard JN, Shiloach J, Segal D, *et al.* Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science 2008;320:379-81.

Liu YP, Wu GS, Yao YG, Miao YW, Luikart G, Baig M, *et al.* Multiple maternal origins of chickens: out of the Asian jungles. Molecular Phylogenetics and Evolution 2006;38:12-9.

Medzhitov R. Toll-like receptors and innate immunity. Nature Reviews Immunology 2001;1:135-45.

Misch EA, Hawn TR. Toll-like receptor polymorphisms and susceptibility to human disease. Clinical Science (London) 2008;114:347-60.

Mukherjee S, Huda S and Sinha Babu SP. Toll-like receptor polymorphism in host immune response to infectious diseases: A review. Scandinavian Journal of Immunology 2019;90:e12771.

O'Dwyer DN, Armstrong ME, Trujillo G, Cooke G, Keane MP, Fallon PG, *et al.* The Toll-like receptor 3 L412F polymorphism and disease progression in idiopathic pulmonary fibrosis. American Journal of Respiratory and Critical Care Medicine 2013;188:1442-50.

Philbin VJ, Iqbal M, Boyd Y, Goodchild MJ, Beal RK, Bumstead N, *et al.* Identification and characterization of a functional alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. Immunology 2005;114:507- 21.

Piaserico S, Michelotto A, Frigo AC, Alaibac M. TLR7 Gln11Leu single nucleotide polymorphism and response to treatment with imiquimod in patients with basal cell carcinoma: a pilot study. Pharmacogenomics 2015;16:1913-7.

Ruan W, Wu Y, Zheng S J. Different genetic patterns in avian Toll-like receptor (TLR)5 genes. Molecular Biology Reports 2012;39:3419-26.

Ruan W, An J, Wu Y. Polymorphisms of chicken TLR3 and 7 in different breeds. PloS One 2015;10:e0119967.

Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, *et al.* The evolution of vertebrate Toll-like receptors. Proceedings of National Academy Science USA 2005;102:9577-82.

Satake H, Sasaki N. Comparative overview of toll-like receptors in lower animals. Zoological Science 2010;27:154-61.

Schott E, Witt H, Neumann K, Taube S, Oh DY, Schreier E, *et al.* A Toll-like receptor 7 single nucleotide polymorphism protects from advanced inflammation and fibrosis in male patients with chronic HCV-infection. Journal of Hepatology 2007;47:203-11.

Swiderska Z, Smidova A, Buchtova L, Bryjova A, Fabianova A, Munclinger P, *et al.* Avian Toll-like receptor allelic diversity far exceeds human polymorphism: an insight from domestic chicken breeds. Science Report 2018;8:17878.

Valanne S, Wang JH and Ramet M. The Drosophila toll signaling pathway. Journal of Immunology 2011;186:649-56.

Velova H, Gutowska-Ding MW, Burt DW, Vinkler M. Toll-like receptor evolution in birds: gene duplication pseudogenisation and diversifying selection. Molecular Biology and Evolution 2018;35(9):2170-84.

Verstak B, Nagpal K, Bottomley SP, Golenbock DT, Hertzog PJ, Mansell A. MyD88 adapter-like (Mal)/TIRAP interaction with TRAF6 is critical for TLR2- and TLR4-mediated NF-kappaB proinflammatory responses. Journal of Biological Chemistry 2009;284:24192-203.

Werling D, Jann OC, Offord V, Glass EJ, Coffey TJ. Variation matters: TLR structure and species-specific pathogen recognition. Trends in Immunology 2009;30:124-30.

Yilmaz A, Shen S, Adelson DL, Xavier S, Zhu JJ. Identification and sequence analysis of chicken Toll-like receptors. Immunogenetics 2005;56:743-53.

Zhang Z, Ohto U, Shimizu T. Toward a structural understanding of nucleic acid-sensing Toll-like receptors in the innate immune system. FEBS Letters 2017;591:3167-81.

Zhang Z, Ohto U, Shibata T, Krayukhina E, Taoka M, Yamauchi Y, *et al.* Structural analysis reveals that toll-like receptor 7 Is a dual receptor for guanosine and single-stranded RNA. Immunity 2016;45:737-48.

Tables 1 to 4:

Table 1 – PCR primers used in this study.

	Primers (5'-3')
avTLR3p1	ATGCTGGAGGAGGTGAAGA
avTLR3p2	TGCAGTCCCTGGAGAGTTAA
avTLR3p3	ACACAGGATGTTTACATGCGATTGG
avTLR3p4	CCCTTGAAAACATGAACTGGAATCTC
avTLR3p5	AAGCAGGAATATCTGAGTTTGAAGC
avTLR3p6	GCATAGTATCTAAACGTTTGTGACCC
avTLR7p1	ATGACAAATCTTTCAGAGGTGGCT
avTLR7p2	GGGGATATGGTTAATAGTCAGGGTC
avTLR7p3	GAAACGCTACTAACCTGACCCTGAC
avTLR7p4	CAGCGTCACCGATCTCCTTTATG
avTLR7p5	CCAAGCAGCTGGTTTAAGAACATCA
avTLR7p6	TCGGGGAACGGTAGTCAGAAGGT
avTLR7p7	GAGCATTCAGCTGAGCAAAAAG
avTLR7p8	CAGTTTCCTGGAGAAGTTTGTTGTA

Table 2 – Avian names and GenBank accession numbers.

No.	0	1	2	3	4	5	6	7	8
Name	Gallus gallus	Acanthisitta chloris	Anser anser	Apteryx australis mantelli	Apteryx rowi	Athene cunicularia	Buceros rhinoceros	Calypte anna	Cariama cristata
TLR3	NM001011691	XM009072290	KC292270	XM013942085	XM026054760	XM026847033	XM010135016	XM008491852	XM009700362
TLR7	NM001011688			XM013950523		XM026856297		XM008494759	XM009696068
No.	9	10	11	12	13	14	15	16	17
Name	Chartura pelagica	Charadrius vociferus	Chlamydotis macqueenii	Colius striatus	Columba livia	Phasianus colchicus	Corapipo altera	Cuculus canorus	Cyanistes caeruleus
TLR3	XM010002467	XM009883901	XM010122732	XM010203505	AB618533	MG604331/2	XM027636061	XM009565656	XM023925083
TLR7			XM010119851	XM010209014	XM005512700	MG604329		XM009555971	
No.	18	19	20	21	22	23	24	25	26
Name	Egretta gazetta	Eurypyga helias	Ficedula albicollis	Fulmarus glacialis	Gavia stellata	Numida meleagris	Haliaeetus albicilla	Haliaeetus leucocephalus	Leptosomus discolor
TLR3	XM009642418	XM010156014	XM005045153	XM009585392	XM009811989	MG604330	XM009915940	XM010570028	XM009953213
TLR7	XM009646337	XM010146638	XM005037400	XM009572361	XM009813692	MG604328	XM009913820	XM010581565	XM009960331
No.	27	28	29	30	31	32	33	34	35
Name	Lonchura striata domestica	Manacus vitellinus	Merops nubicus	Mesitomis unicolor	Neopelma chrysocephalim	Nestor notabilis	Nipponia nippon	Nothoprocta perdicara	Opisthocomus hoazin
TLR3	XM021556089	XM018069963	XM008943939	XM010181262	XM027676546	XM010013832	XM009474837	XM026033588	XM009940192
TLR7	XM021528936	XM008932256	XM008935272			XM010019975	XM009476283	XM026047305	
No.	36	37	38	39	40	41	42	43	44
Name	Passer domesticus	Pelecanus crispus	Phaethon lepturus	Phalacrocorax carbo	Picoides pubescens	Pseudopodoces humilis	Pterocles gutturalis	Struthio camelus	Sturnus vulgaris
TLR3	GU229788	XM009486220	XM010281742	XM009505917	XM009902217	XM014263168	XM010074246	XM009676700	XM014889663
TLR7	KF212180	XM009489888	XM010290259	XM009501983		XM014252874	XM010076693	XM009683661	XM014894280
No.	45	46	47	48	49	50	51	52	53
Name	Taeniopygia guttata	Tauraco erythrolophus	Tinamus guttatus	Tyto alba	Caprimulgus carolinensis	Zonotrichia albicollis	Anas platyrhynchos	Anser cygnoides	Balearica regulorum gibbericeps
TLR3	XM002190852	XM009986371	XM010219604	XM009970053	XM_010170240	XM005483864			
TLR7		XM009991025		XM009975060	XM_010175260		XM005029176	KJ022638	XM010309901
No.	54	55	56	57	58	59	60	61	62
Name	Parus major	Corvus cornix cornix	Coturnix japonica	Dromaius novaehollandiae	Empidonax traillii	Falco cherrug	Falco peregrinus	Serinus canaria	Pavo cristatus
TLR3									
TLR7	XM015629850	XM019283098	AB553582	XM026116653	XM027905403	XM027798644	XM027785411	XM018913537	KX712249
No.	63								
Name	Pygoscelis adeliae								
TLR3									
TLR7	XM009318873								

Table 3 – Avian TLR3 differences sites at functional region.

Site	Stru	Aa (codon)	Avian number	Aa (codon)	Avian number
57	LRR	Q(CAA)	0/2-4/27/34/39/40/47	E(GAA)	1/5-12/15-22/24-26/28-31/33/35/37/38/41/42/44-46/48-50
59	LRR	H(CAC)	0/2-4/17/34/38-41/46/47	Y(TAT)	1/5-8/10/12/16/18-22/24-26/29/30/32/33/35/37/42/44/48-50
60	LRR	E(GAA)	0/2-4/34/39/47	K(AAG)	1/5-12/15-22/24-33/35/37/38/40/41/44-46/48-50
64	LRR	G(GGA)	0/2/8/12/24/35/37-42/44-50	R(AGA)	1/3-7/9-11/15-18/20-22
93	LRR	E(GAG)	0-2/5-12/15-19/21-22/24-26/28-31/33/35/37-42/46/48/49	K(AAG)	3-4/20/27/32/34/44/45/47/50
112	LRR	L(TTG)	0/1/5-11/15/16/19-22/24-33/37-42/44/45/48-50	S(TCG)	3-4/17/46
117	LRR	K(AAG)	0/1/3-12/15/16/18/19/21/22/24-26/28/29/31-34/37-40/42/46-49	E(GAG)	17/20/27/35/41/44/45/50
118	LRR	N(AAC)	0-2/5/7-12/15/16/18/19/21/22/24-26/28-33/35/37-40/42/46/48/49	K(AAA)	6/17/20/27/41//44/45/50
120	LRR	R(CGT)	0/2/5/7-11/18/19/21/24/25/29/30/32/33/35/37-40/42/44/46	H(CAT)	1/3/4/12/15-17/20/22/26-28/31/34/41/45/47-50
123	LRR	V(GTG)	0/2-4/34/35/39/47-49	M(ATG)	1/5-12/15-21/26-33/37/38/41/42/44/45/46/50
163	LRR	L(TTA)	0-6/8/10-12/15/16/18/19/21/22/24-26/28-35/37-40/46/47/49	F(TTC)	7/9/17/20/27/41/42/44/45/48/50
165	LRR	A(GCG)	0/2-12/16/18/19/21/22/24-26/29/30/32-35/37-40/42/46-49	T(ACA)	1/15/17/27/28/31/41/44/45/50
224	LRR	V(GTT)	0/2/5-12/14/16/18/19/21-26/29/30/32-35/37-40/42/46/47/49	I(ATT)	1/15/17/20/27/28/31/41/44/45/48/50
229	LRR	K(AAA)	0/2-4/14/23/34/39/47	Q(CAA)	1/5-12/15-20/22/24/25/27/28/30-33/35/37/38/41/42/44-46/48-50
237	LRR	D(GAT)	0-8/11-12/14-16/18/19/21-26/28-33/35/37-40/42/46-49	N(AAT)	10/17/20/27/41/44/45
247	LRR	E(GAG)	0/2-4/14/23/34/39/47	Q(CAA)	1/6-11/15/16/18-22/24/25/27-32/35/37/38/40/42/45/46/48-50
252	LRR	E(GAG)	0/6/14/23/30/39/46-50	K(AAG)	1-2/5/7-8/10/12/15-22/24-29/31-33/35/37/38/40-42/45
253	LRR	D(GAT)	0/2-4/14/23/26/34/39/40/47	H(CAT)	1/5-12/15/16/21/22/24/25/28-33/35/37/38/42/46/48/49
262	LRR	H(CAT)	0/3-4/8/33/39	R(CGT)	1-2/5/7/9/10/12-15/17-32/37/38/40/41/44-46/48/50
292	LRR	H(CAC)	0/26/14/39/47	Y(TAC)	1-6/8/9/12/15-25/27-33/35/37/38/40-42/45/46/48-50
381	LRR	N(AAT)	0/1/5/14/17/23/35/39/46	S(AGT)	2/6-12/15/16/18-21/24-29/31-33/37/38/40-42/44/45/48-50
383	LRR	E(GAG)	0/2-4/6-12/14/16/18/19/21-26/29/30/32-35/37-39/42/46/47/49	Q(CAG)	1/15/17/20/27/28/31/40/41/44/45/50
443	LRR	R(CGG)	0/10/14/15/17/19/20/27/39/41/44/49/50	Q(CAA)	1-9/11-12/16/18/21-26/28-35/37/38/42/45-48
459	LRR	R(AGG)	0/7/9/12/24/25/29/39	K(AAA)	1/3-6/8/10/11/14-23/26-28/30/32/34/35/37/38/40-42/44-49
460	LRR	E(GAA)	0-2/5-10/14/15/17/19-28/30-33/35/37-41/44/45/48-50	D(GAT)	3-4/12/16/18/34/46/47
485	LRR	C(TGT)	0/31/32/34/35/37-42/44-50	H(CAT)	1-5/7/8/11/12/15-18/20-22/24-29
511	LRR	F(TTT)	0/2-4/34/39/41/47	L(CTT)	1/5-9/11/12/15-22/24-28/30-33/38/40/42/44-46/48/50
603	LRR	A(GCA)	0/2-12/16-18/21/24-26/29/30/32/33/37-39/41/42/45-50	V(GTA)	1/15/20/22/27/28/31/44
604	LRR	Y(TAT)	0/2-6/8-12/16/18/19/21/22/24-26/29/30/32-35/37-40/42/46-49	D(GAT)	17/27/28/31/41/44/45/50
609	LRR	Q(CAG)	0-4/12,24-26/29/32/34/39/40/47	R(AGG)	5/7/9-11/16-18/20-22/27/30/33/37/38/41/42/44-46/50
627	LRR	H(CAT)	0/2-12/16/18/19/21/22/24-26/29/30/32-35/37-40/42/46-49	Y(TAT)	1/15/17/20/27/28/31/41/44/45/50
705	TIR	N(AAT)	0-2/5/6/8/11/15/17/19-22/24-29/31-33/35/38-42/44-46/48-50	D(GAT)	3/4/9/10/16/18/30/34/37/47
716	TIR	E(GAA)	0/3/4/12/16/24/25/32/34/39/46/47	K(AAA)	1/2/5-11/15/17-22/26-31/33/35/37/38/40/41/44/45/48-50
719	TIR	Q(CAA)	0/2-5/16/39	H(CAC)	6-9/11/15/17-22/24-31/33/35/37/38/40/41/44/45/48-50
721	TIR	Q(CAA)	0/3-9/11/16/18/19/21/22/24-26/29/33/35/37-39/46/48/49	E(GAA)	1/2/15/17/20/27/28/31/41/44/45/50
723	TIR	K(AAG)	0/1/7/9/10/12/17/20/26/27/34/38-41/44/45/47/50	R(AGG)	2-6/8/11/15/18/19/ 21/ 22/ 24/25/ 28/ 29/ 31/33/35/37/48/49
735	TIR	I(ATA)	0-5/7-10/12/16/18/21/22/26/29/30/32-35/37/39/40/42/48/49	V(GTA)	6/11/17/19/20/24/25/27/38/41/44/45/50
741	TIR	I(ATA)	0/2/7/9/39/46/47/49	T(ACA)	1/3-6/8/10/12/15-22/24--35/37/38/40-42/44/45/48/50
746	TIR	R(AGA)	0/2/7/30/34/39/40/47	K(AAA)	1/3-6/8-10/12/15-22/24-29/31-33/35/37/38/41/42/44-46/48-50
747	TIR	R(AGG)	0/2-4/20/29/34/39/40/44/47	M(ATG)	1/6-12/15-19/21/22/26-28/31-33/35/37/38/41/46/48-50
767	TIR	K(AAG)	0/10/15-22/24/28/30/31/33/39/46	N(AAT)	1/5-8/11/12/25-27/29/32/35/37/41/42/44/45/48-50(9/40
799	TIR	N(AAT)	0/2-4/34/37-39/41/42/47	Y(TAT)	1/5-9/12/15-19/21/22/24-31/33/35/44-46/48-50
803	TIR	C(TGC)	0/2-4/39/47	H(CAC)	5-10/12/15-1921/22/24-28/31-33/35/38/40-42/45/46/48-50
810	TIR	R(AGA)	0-3/5/6/8/10-12/15/17/20/24-32/34/35/38-42/44-48/50	K(AAA)	4/9/16/18/19/21/22/33/37/49
812	TIR	C(TGC)	0/15/24/25/28/31/39	R(CGC)	2/3/6/9/10/18-22/26-27/29/30/32-35/37-38/40-42/44-50

Abbreviations: Stru (structural character), Aa (amino acid), LRR (leucine-rich repeat domain), TIR (Toll/interleukin-1 receptor domain)

Table 4 – Avian TLR7 differences sites at functional region.

	Stru	Aa (codon)	Avian number	Aa (codon)	Avian number
Site					
51	LRR	F(TTT)	0/14/23-25/32/34/36/48/56/62/63	S(TCT)	5/7/8/11-13/16/18/19/21/22/26/29/33/37-39/42/46/49/51-53/59/60
56	LRR	R(AGA)	0/14/23/36/48/51/52/56/62	T(ACA)	3/5/7/8/11/13/18-22/24-29/32/33/37-39/41-43/49/53-55/58-61/63
60	LRR	S(AGT))	0/3/14/36/43/48/56/57/62	T(ACT)	7/13/20/26/29/32/38/39/41/42/44/46/49/53-55/59-61
135	LRR	S(TCA)	0/8/36	T(ACA)	5/7/11/13/18/19/21-29/33/34/37-39/41-44/46/48/49/51-63
149	LRR	K(AAA)	0/36	E(GAA)	3/5/7/8/11-13/16/18-29/32-34/37-39/41-44/46/48/51-63
152	LRR	R(CGG)	0/3/5/12/13/22/23/26/29/32-34/36/38/48/51/52/56/57/62	Q(CAG)	7/8/14/18/19/21/24/25/37/39/42-43/49/53/63
159	LRR	R(CGT)	0/3/14/26/34/36/42/43/56/57/62	S(AGT)	5/7/11-13/16/18/19/21-25/29/32/33/37-39/46/48/49/51-53/59/60/63
175	LRR	T(ACT)	0/14/23/36/56/58/62	N(AAT)	3/7/8/13/16/18/19/21-22/24-26/29/32-34/37-39/42/43/ 46/48-49.51-53/57/63
176	LRR	F(TTT)	0/7/14/23/36/56/62	L(TTG)	3/5/11-13/16/18/19/22/24/25/28/29/32/34/37/38/42/43/46/48/53/57-60/63
213	LRR	N(AAT)	0/3/14/23/34/36/43/51/52/56-58/62	K(AAA)	5/7/8/11/13/16/18/19/21/22/24-26/28/32/33/37-39/ 42/46/48/49/53/59/60/63(12/20/27/29/41/44/54/55/61
216	LRR	V(GTA)	0/14/23/26/33/3652/56/62	I(ATA)	3/5/7/8/11-13/16/18-22/24/25/27/28/32/34/37-39/41-44/46/48/53-55/57-60/63
244	LRR	R(AGA)	0/12/14/23/27/28/36/56/62	M(ATG)	3/5/7/8/11/13/16/19-22/24-26/29/33/37-39/41/42/46/48/49/53-55/57/59-61/63
247	LRR	E(GAG)	0/5/14/16/20/23/27/34/36/41/44/51/52/54/56/61/62	V(GTG)	3/7/8/11-13/18/19/21/22/24-26/28/29/32/33/37-39/42/43/46/48/49/55/57-60/63
248	LRR	V(GTT)	0/13/14/23/36/39/51/52/56/62	I(ATT)	3/5/7/8/11/12/16/18-22/24-29/32-34/37/38/41-44/46/48/49/53-55/57-61/63
298	LRR	K(AAA)	0/14/34/36/41/51/52/54/56/62	N(AAT)	5/7/8/11-13/16/18-22/24-26/28/29/32-33/37/39/42/44/46/48/49/53/55/58-61/63
321	LRR	K(AAG)	0/5/14/20/23/27-29/36/44/48/55-58/61/62	R(AGG)	3/7/8/11-13/16/18/21/22/24-26/32/33/37-39/41-43/46/49/51-54/59/60/63
341	LRR	E(GAG)	0/14/20-29/32-34/36-39/41/42/54/59/60/62	Q(CAG)	3/5/7/8/11-13/16/18/19/43/44/46/49/51-53/55-58/61/63
418	LRR	I(ATT)	0/7/14/16/20/33/41/53/54/56/62	V(GTT)	3/5/8/11/12/18/19/21-29/32/34/36-39/42-44/46/48/49/51/52/55/57-61/63
428	LRR	R(CGA)	0/18	P(CCA)	3/5/7/812/13/16/19-22/24-29/32-34/36-38/41-44/46/48/49/53-55/57-61/63
429	LRR	S(TCC)	0	A(GCT)	3/8/11-14/16/18-22/24/25/28/29/32-34/36-39/41-44/48/49/51-55/57/59-61/63
432	LRR	I(ATC)	0/14/23/62	L(CTC)	5/7/8/11-13/19/24/25/29/38/42/46/49/53/59/60
437	LRR	M(ATG)	0/24-26/29	V(GTG)	3/5/7/11-14/16/19-23/27/28/32-34/36-39/41-44/48/49/51-63
445	LRR	G(GGT)	0/3/5/8/14/16/18/21-26/32/33/38/39/42/43/48/49/53/56/57/62	S(AGT)	19/46/51/52/59/60/63
528	LRR	F(TTC)	0/8/11/12/14/21-25/33/34/36/37/43/ 46/49/51-53/56/57/62/63	S(TCC)	5/13/16/18/20/26-29/38/41/42/44/48/54/55/59-61
530	LRR	K(AAA)	0/7/12/14/20/23/27/36/37/41/44/54-56/61/62	R(AGA)	3/5/8/11/13/16/19/21/22/24-26/29/32-34/38/42/43/46/48/49/51-53/57-60/6
573	LRR	K(AAA)	0/3/8/13/14/18-25/27-29/33/34/36-39/41-44/48/53-56/58/61-63	E(GAA)	16/26/46/49/51/52/57/59/60
596	LRR	S(AGC)	0/14/36/49/56/62	T(ACT)	5/7/8/11/13/16/18/19/22/24-29/38/39/41-43/46/48/51/52/54/55/58/61/63
661	LRR	N(AAT)	0/23/36/39/56/62	S(AGC)	5/7/8/11-13/16/18/20-22/24-29/32-34/37/38/41-43/46/48/49/53-55/57-60/63(3)
664	LRR	Q(CAA)	0/3/5/7/8/11-13/16/18/19/21-26/29/33/36/37/39/42/43/46/48/49/52/53/56/59/60/62/63	E(GAA)	20/27/28/32/34/41/44/54/55/57/58/61
671	LRR	M(ATG)	0/14/23/36/56/62	S(TCA)	7/8/13/16/20/24/25/27/29/32/37/41/42/44/49/51/52/54/59/60/61
689	LRR	I(ATA)	0/3/14/34/36/43/51/52/57/62	V(GTC)	12/13/29/46/59/60
694	LRR	S(AGT)	0/3/14/23/36/56/58/62	N(AAT)	7/8/11/13/16/18-22/24-29/33/34/37/39/41-44/46/48/49/51-55/57/59/60/63
697	LRR	L(TTG)	0/12/14/23/28/36/43/51/52/56/58/61/62	M(ATG)	5/7/8/16/19/22/24-26/29/32/37-39/42/46/48/53/63
698	LRR	H(CAC)	0/36	K(AAG)	3/5/7/11-14/18-29/32/33/34/37-39/41-44/46/48/49/51-63
712	LRR	I(ATA)	0/3/11/36/43/57/62	V(GTA)	5/8/12-14/18/20-29/33/37/38/41/42/44/46/48/49/53-56/58-61/63
726	LRR	R(CGA)	0/3/7/13/14/16/18/22-26/28/29/33/36/37/39/41/42/46/48/49/51-54/56-58/61/62	Q(CAA)	5/8/12/21/27/32/38/43/59/60/63
727	LRR	K(AAG)	0/14/23/36/62	E(GAA)	3/5/7/8/11/-13/16/18-22/26/28/29/32-34/37-39/41-44/48/49/51-55/57-61/63
732	LRR	T(ACT)	0/14/20/23/27/36/41/44/51/52/54/56/62	S(TCT)	3/5/7/8/11-13/16/18/19/21/22/24-26/29/32-34/37-39/42/43/46/48/49/53/57-61/63
739	LRR	I(ATA)	0/3/7/14/23/34/36/56/57/62	M(ATG)	5/8/11/-13/16/18-22/24-29/33/37-39/42/43/46/48/49/53/55/58-61
744	LRR	R(CGT)	0/14/20/23/29/36/57/58/62/	H(CAC)	18/32-34/37/51/59/60
748	LRR	I(ATA)	0/14/23/36/44/62	L(CTA)	3/7/8/11/-13/16/18/19/21/22/24-29/32/34/37-39/41-43/46/48/49/51-54/57-60/63
751	LRR	Y(TAT)	0/7/13/14/20/23-27/36/44/46/51/52/55/56/61/62	H(CAT)	3/5/8/11/12/16/18/19/21/22/28/29/32-34/37-39/41-43/48/49/53/54/57-60/63
757	LRR	I(ATT)	0/14/23/36/44/51/52/56/62/	F(TTT)	3/5/7/8/11-13/16/18-22/24-29/32-34/37-39/41-43/46/48/49/53-55/57/58/61/63
771	LRR	I(ATA)	0/3/5/7/8/13/14/18/19/21/22/26/27/29/33/34/36/37/39/41-44/48/49/51-57/59-63	V(GTA)	12/20/23-25/28/46

Abbreviations: Stru (structural character), Aa (amino acid), LRR (leucine-rich repeat domain).