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On the partial *TLR21* gene from the feathers of birds, a chicken, and a duck

The evolutionary arms race between pathogens and host organisms has shaped diverse immune detection systems across species. Among the most studied innate immune components are Toll-like receptors (TLRs), which bridge environmental signals with host immune responses (El-Zayat *et al.* 2019). Originally discovered in *Drosophila melanogaster* as a regulator of embryonic development, the Toll gene was later found to have antifungal immunity functions. This laid the foundation for the discovery of vertebrate TLRs, including those in birds. TLRs recognize structurally conserved molecules of microbes, known as pathogen-associated molecular patterns (PAMPs), encompassing lipids, lipoproteins, proteins, and nucleic acids (Rehman *et al.* 2021). Avian TLRs are structurally conserved with their mammalian counterparts but display unique features, such as the presence of TLR21 in birds, which functionally resembles mammalian TLR9 by detecting unmethylated CpG motifs (Lai *et al.* 2019). This study focuses on detecting and characterizing the partial cDNA of TLR21 from six Indonesian endemic/endangered avian species, such as the Java sparrow, using feather samples. By employing non-invasive methods, this study opens avenues for immunogenetic research without harming the animals, especially critical for conservation efforts.

Feather samples were collected non-invasively from six avian species: KUB-1 native chicken (*Gallus gallus domesticus*), Alabio duck (*Anas platyrhynchos Borneo*), zebra dove (*Geopelia striata*), spotted dove (*Streptopelia chinensis*), ring dove (*Streptopelia risoria*), and Java sparrow (*Lonchura oryzivora*). Ethical clearance was obtained from the Ethics Commission of Institut Teknologi Bandung (02/KEPHP-ITB/10-2019). Total RNA was extracted from the calamus of downy feathers using the RNeasy Mini Kit (Qiagen), followed by reverse transcription using AccuPower CycleScript RT Premix. PCR amplification targeted a 148 bp region of TLR21 using species-conserved primers. Amplicons were visualized on 2% agarose gel, purified, and sequenced by First Base (Singapore). Sequence editing and alignment were conducted using Chromas, BioEdit, and Clustal Omega. Homology analysis was performed using BLAST, while protein translation was performed using the ExPASy tool. A phylogenetic tree was constructed using MEGA7 via the Neighbor-Joining method with 1,000 bootstrap replicates, incorporating zebrafish (*Danio rerio*) TLR21 as an outgroup.

Electrophoresis revealed a distinct 148bp band for five of the six avian species (excluding zebra dove, whose sequencing was inconclusive). Java sparrow's electropherogram showed two bands, but only the target 148 bp band was analyzed further. BLAST analysis confirmed ~99% homology of the partial TLR21 cDNA with reference sequences (NM_001030558.3 and NP_001025729.3), except for the Alabio duck, which showed ~96.7% homology. Translation of cDNA yielded 31 amino acid fragments aligning with the partial TLR21 protein of *G. gallus*. Phylogenetic analysis grouped KUB-1 chicken closely with Broiler chicken, while Alabio duck and the other doves formed separate branches. The inclusion of zebrafish TLR21 confirmed the ancestral linkage of this gene within non-mammalian vertebrates.

This study successfully demonstrates the expression of TLR21 in the feathers of several Indonesian avian species using non-invasive sampling. This approach is particularly valuable

for research on vulnerable or endangered birds like the Java sparrow, where preserving life is paramount. Despite its short sequence length, the 148 bp fragment proved informative for phylogenetic comparison, supported by previous studies (Asvapathanagul & Olson 2017). Short conserved sequences are often sufficient to infer evolutionary relationships when full-length sequences are unavailable or impractical to obtain. The detection of TLR21 in feathers also underscores the potential of feathers as a reliable source of immune-related genetic material. Feather calamus, particularly during growth phases, contains rich cellular material conducive to RNA extraction (Brown *et al.* 2022). The pulp within the feather follicle has been shown to mirror immune responses in lymphoid tissues, contributing valuable information for studies in avian immunology (Schat & Skinner 2022). Evolutionary analysis revealed close homology between domestic chicken breeds and partial divergence among ducks and doves. This finding supports previous observations on the paraphyletic nature of avian TLR21 evolution (Suryohastari *et al.* 2023). The lack of mammalian orthologs further reinforces the uniqueness of TLR21 in birds, reptiles, and fish, with functional similarities but divergent evolutionary histories (Zhang *et al.* 2014). While the Zebra dove sequence could not be included due to low-quality reads, future studies may attempt higher coverage or alternative primer sets to address this gap. Furthermore, a more complete understanding of TLR21 function and evolution in birds would benefit from full-length sequencing, functional assays, and expression profiling across developmental stages and tissues.

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