

Genetic Diversity at Toll Like Receptor 7 (TLR7) Gene of Sri Lankan Indigenous Chicken and Ceylon Jungle Fowl (*Gallus lafayetti*)

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ABSTRACT. Although the Sri Lankan indigenous chicken is providing a valuable protein source and genetic resource to the country, a systemic evaluation and proper characterization of indigenous chicken population remains at a zero level. Here we use experimental molecular tool to identify the variation of indigenous chicken at immune related gene Toll like receptor 7 (TLR7). A total of 24 Single Nucleotide Polymorphism (SNPs) and 35 haplotypes were identified within Sri Lankan Indigenous chicken with relative frequencies of 0.0208 to 0.0625. The genetic structure of indigenous chicken populations showed a relatively low genetic distance (overall average 0.698). Majority (about 80%) of the population were identified in one large cluster in phylogenetic analysis, confirming the fact that being in the family of Toll Like Receptors, TLR7 locus is a conserved region among the indigenous chicken population. However, the effect of SNPs in the protein coding region, gene expression and relation to disease resistance found in this study is being investigated.

Key words: Genetic diversity, Indigenous chicken, Single Nucleotide Polymorphism (SNPs), Toll like receptor 7 gene.

INTRODUCTION

More than 10,000 years ago, during the transition of humans from hunting to manipulation of the surroundings for their benefit, the process of domestication began. Chicken (*Gallus gallus domesticus*) is considered to be one of the first domesticated animals and it represents one of the most important sources of food protein worldwide. It is believed that domesticated chicken originated from Red Jungle fowl (*Gallus gallus*). From the beginning of the domestication process, the exchange and transportation of domestic animals take place in relation to human migration and trade. This resulted in genetic and geographic variation in global distribution of different breeds or geo-types of *Gallus gallus*.

Since the domestic chicken has been distributed in various geographic locations with different feeding and breeding regimes, they show various levels of adaptations. These encompass more or less unselected indigenous chicken and ecotypes from various regions in the world (Downing *et al.*, 2009). The genetic characterization of these populations using molecular tools is useful to help conserve the valuable genetic variation they possess.

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Sri Lankan indigenous or village chicken contributes approximately 15% to the egg production in Sri Lanka (Chandrasiri and Gamage, 2002). The distribution of village chicken population in the country is highly variable since they are one of the most important and geographically wide spread livestock species in the country. The origin of the indigenous chicken of Sri Lanka is not clear. However, as in other indigenous livestock species in the country, chicken types are also believed to be carrying various levels of contribution from different genotypes brought to the country in the past. Therefore, having originated as a blend of different genotypes, adapted to local climate and feeding regimes through centuries, the indigenous chicken form a unique genetic resource for the country (Silva and Premasundara, 2002). However, with the pressure of high producing breeds and the continuous introduction of exotic breeds within the policy framework of increasing the production, and in the absence of due consideration for the values and roles of indigenous animal genetic resources has led to the erosion of valuable genetic resources (Silva and Premasundara, 2002). As a result, the well adapted indigenous types are facing a threat of extinction at present.

In general, there is no proper characterization or systematic evaluation carried out on these valuable genetic resources. The future utilization of indigenous genetic resources depends on their proper evaluation and conservation through upgrading of productivity. The use of molecular tools to assess genetic characteristics is the key component in the strategy for conservation of genetic resources of indigenous animals for sustainable utilization.

Immunity as a genetic tool for characterization

Although chicken represents a valuable genetic resource and protein source, it remains a potential threat to human health as they serve as a reservoir for diseases and food borne pathogens (Xing *et al.*, 2008). Emerging diseases such as avian influenza provide a new impetus to investigate chicken immunity, in particular, the relationship between population diversity and disease susceptibility (Downing *et al.*, 2009). On the other hand, identification of a biologically meaningful, immunity related gene which causes variation among populations will provide a key element in characterization and further studies of disease resistance traits.

The geographic distribution, population densities and disease epidemiology of chicken are likely to have changed dramatically since their domestication and undoubtedly re-molded their genetic diversity. Novel diseases and increased incidence of infection would have challenged the chicken immune response, necessitating adaptive evolution at key immune genes (Downing *et al.*, 2009). Evidence for such adaptation is found in the sequence conservation of immunity related genes (International Chicken Genome Sequencing Consortium, 2004), and in several studies, the association of allelic variation at particular immune genes with susceptibility to infection has been reported. Genes involved in the immune system, therefore, could be an appealing candidate for examining the selective processes shaping genetic diversity (Downing *et al.*, 2009) and can provide insight into the complex relationship between diseases and genes (Akey *et al.*, 2004).

The toll like receptor (TLR) gene family is a highly conserved group of DNA molecules (Boyd *et al.*, 2007) which perform a vital role as sentinels of the innate immune system in their host organism through the recognition of Pathogen Associated Molecular Patterns (PAMPs) (Temperley *et al.*, 2008). They are triggered by antiviral compounds and single-stranded RNA, and are implicated in immune response to viruses such as influenza (Boyd *et al.* 2007).

TLR genes are highly conserved domains present in a wide range of taxa of plants and animals (Temperley *et al.* 2008). With evolutionary evidence it is believed that, avian and mammalian lineages diverged from a common ancestor approximately 300 million years ago. The avian TLR repertoire comprises both orthologous and distinct TLR genes (Boyd *et al.* 2007). These receptors have been described in many animal phyla and the genomes of most vertebrate species encode between 10 and 13 different TLRs (Boyd *et al.* 2007), where TLR7 is one member of the group.

Gene gain and gene loss are important evolutionary processes, especially with respect to gene family dynamics (Cotton and Page, 2005). Gene duplication is believed to be the principal cause by which new genes are created (Temperley *et al.* 2008). Even though TLR 7, located on chromosome number one, is a highly conserved sequence, it accepts some mutations. For example, point mutations/ single nucleotide differences/ SNPs will create variation among populations of the same species. Since the TLR7 gene is characterized and sequenced, it allows analysis of variation among Sri Lankan indigenous Chicken and Jungle fowl at TLR7 gene. The present study is an attempt to characterize the native chicken population, and the endemic wild-relative in Sri Lanka, using TLR7 gene.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Venous blood samples were collected from a total of 150 birds including six Ceylon Jungle Fowl (CJF) and indigenous village chicken representing five provinces [North Central – 19 (SL-NC), North Western - 17 (SL-NW), Southern - 37 (SL-SP), Western - 20 (SL-WP) and Uva - 37 (SL-UP)]. Sampling was done in representative households in villages scattered in each province to capture the maximum variation. Samples were collected on Whatman FTA[®] filter paper (Whatman Bio-Science, Maidstone, UK) and were labeled separately for laboratory investigation. Genomic DNA from the FTA filter paper was extracted using the method described by Smith and Burgoyne (2004).

Identification of candidate gene TLR7 and SNP Analysis

A total of 75 samples were randomly selected (15 samples from each province and two CJF) for TLR7 gene discovery and SNP analysis. A PCR based sequencing method was employed to genotype the candidate gene and SNPs using the facilities available at CAAS-ILRI joint lab, Beijing, China. Exon 2 region was amplified with predesigned primers (Table-1) with Touchdown PCR using Applied Biosystems thermo cycler. Denatured PCR products were separated using Single-Stranded Conformation Polymorphism (SSCP) method (Li *et al.*,2006) and the distribution of alleles assessed according to single stranded fragment movement. Direct sequencing was carried out for selected PCR fragments for SNP analysis.

Table 1. Primers and sequences used to amplify TLR7 Exon 2 region

Primer	Primer sequence
Tlr7-2F	CAT TTC TGG CCA GTG TGA GAC AG
Tlr7-2R	TTC ATG CCT CTC TAA CCT GGA AAC

Statistical analysis

The sequences were edited and assembled using Chromas (Version 2.33) software and sequences were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) version 4 software (Tamura *et al.*, 2007). Haplotype analysis was carried out using an Integrated Software Package for Population Genetics Data Analysis, Arlequin version 3.5.1.2 (<http://cmpg.unibe.ch/software/arlequin35/>)

RESULTS AND DISCUSSION

This investigation confirms the presence of conserved TLR7 gene in Sri Lankan indigenous chicken genome. About 53% of the total sample was experimentally scanned for TLR7 gene. The target region is about 450 bp in length. A total of 24 Single Nucleotide polymorphism (SNPs) sites were identified. Of the total number of SNPs, three closely located SNP loci (332bp, 340bp, and 374 bp) were found at high frequency, where two SNPs were transitions (G-A and A-G substitution) and one transversion (A-T substitution).

Investigations done using SSCP and direct sequencing identified 35 haplotypes among Sri Lankan indigenous chicken and CJF samples. Haplotype frequency estimations were carried out at intra-population level (Fig. 1) and 3 haplotypes were found at higher frequencies (0.0625) and majority of haplotypes showed low relative frequencies (0.0208).

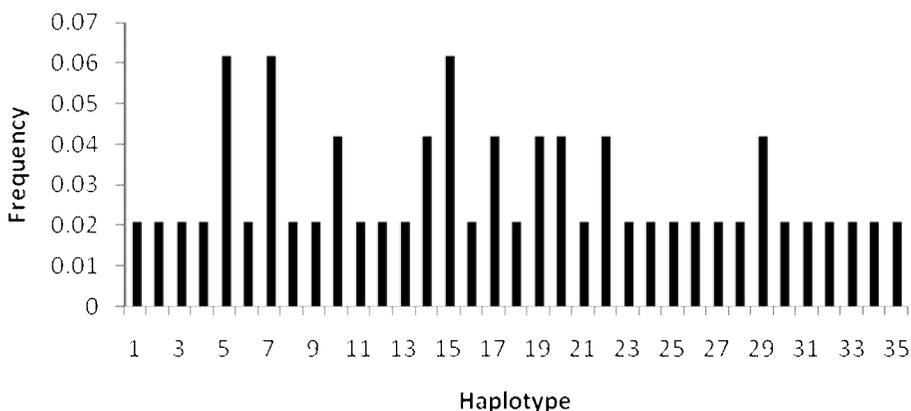


Fig. 1. Haplotype frequency distribution of TLR 7 in Sri Lankan indigenous chicken and native Ceylon jungle fowl

The distribution of haplotypes in different geographical regions was estimated (Fig. 2) and it was found that some haplotypes were shared among populations whereas some were unique within the population (Table 2). The CJF were detected only with haplotypes 2 and 3, which were unique. The highest numbers of haplotypes were found in SL-UP chicken population and least were found with CJFs. This observation confirms that CJF carries highly conserved region at TLR7 compared to domesticated chicken. Given the fact that haplotypes 2 and 3 are unique to CJF and not found in any domesticated chicken proves that CJF has a different evolutionary path to domestic chicken. Further, it reveals that there is no introgression between the two populations.

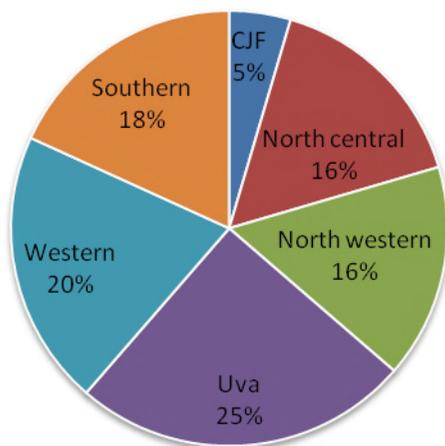


Fig. 2. Distribution of haplotypes in different geographical regions

Table 2. The distribution of haplotypes in different geographical regions

Population	Haplotypes
Ceylon Jungle fowl	2,3
North central chicken	4,5*,6,7*,8,9,10
North western chicken	5*,11,12,13,14,15*,16
Uva province chicken	5*,15*,17*,18,19*,20*,21,22*,23,24,25
Western province chicken	17*,19*,20*,22*,26,27,28,30
Southern province chicken	7*,10*,15*,31,32,33,34,35

* Haplotypes found in more than one population

A relatively low genetic distance (overall average of 0.698) was observed when the genetic structure of the indigenous chicken population was analyzed with respect to TLR7 gene. This confirms that the TLR7 is a highly conserved domain among indigenous chicken to a certain extent, where variation is minimum. The observed low level of variation may be due to isolated breeding in certain geographical regions. This is further depicted in phylogenetic tree analysis (Fig. 3) where the chicken samples from one region have been placed close together. The CJF, which is a progenitor species endemic to Sri Lanka, shows a considerable genetic distance from indigenous village chicken. This again suggests that indigenous chicken in Sri Lanka have been evolved separately from the Ceylon Jungle fowl.

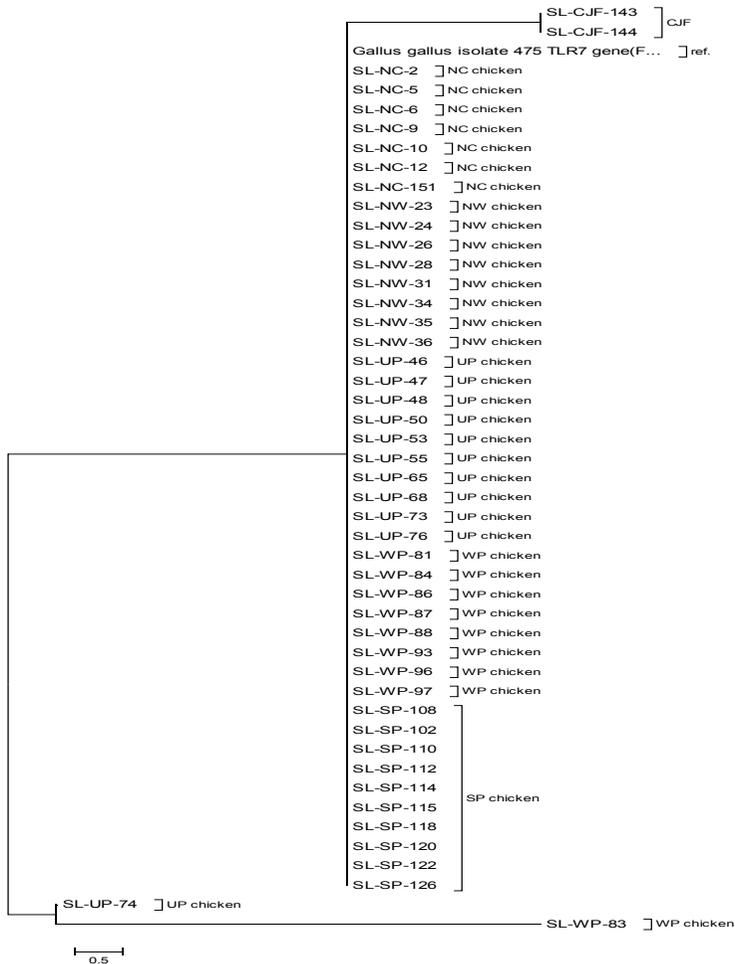


Fig. 3. Phylogeny of TLR7 sequence using neighbor- joining method of Sri Lankan indigenous village chicken (SL-NC, SL-NW, SL-UP, SL-WP, SL-SP) and Ceylon Jungle fowl (CJF)

Phylogenetic investigation of TLR7 gene was performed using Neighbour- Joining (NJ) method (Fig. 3). Phylogenetic tree shows a main cluster representing more than 80% of the samples. As expected, Ceylon Jungle fowl was found with a different cluster falling away from indigenous village chicken. There were two outlier samples.

CONCLUSIONS

Comparative sequence analysis and phylogenetic analysis of Sri Lankan indigenous chicken and Ceylon jungle fowl showed that indigenous chicken and the endemic wild-relative has a distant relationship. The TLR 7 region is highly conserved in indigenous chicken populations though there are SNPs recorded in the coding region. The influence of SNPs found in protein coding region on disease resistance of these populations is yet to be identified

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