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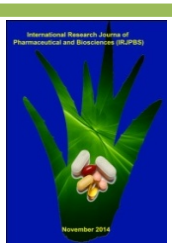

Phylogenetic relationship among red jungle fowl subspecies based on mitochondrial D loop region variability

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Abstract

We studied the nucleotide sequence variation in first 400 bp of D loop region, also known as hyper variable region in Indian red jungle fowl (*Gallus gallus murghi*) and other subspecies of red jungle fowl to establish phytogenetic relationship among them. Out of 400 sites about 12 % were polymorphic between the *Gallus gallus (G.g.)* subspecies and most of them (60%) were transitions. Indian RJF showed very low genetic distances (0.008-0.013) with *G.g. gallus* birds from Thailand as compared to those from Japan and Indonesia. The *G.g. murghi* showed comparatively high genetic distance i.e. 031-0.034 with *G.g. spadiceus* and from 0.061-0.066 with *G. gallus bankiva*. The phylogenetic tree showed that *G.g. banikva* is well separated from the other three subspecies as it made a separate cluster. *G.g. gallus* makes two separate clusters i.e. one those from Thailand and other those from Japan and Indonesia. The *G.g. murghi* falls in one sub-cluster along with the *G.g. gallus* from Thailand. In same cluster, all the *G.g. spadiceus* made one separate sub-cluster.

Key words: D loop, red jungle fowl, polymorphism, phylogeny

INTRODUCTION

Domestication of chickens seems to have a long history as the remains of domesticated chickens from 16 neolithic sites along the yellow river in Northeast China reveals the divergence of about 8000 years between domestic fowl and its ancestor [1]. However, very

strong indication of domestication of chicken was found in the Mohenjo-Doro in the Indus valley [2]. The signs of domestication were also found in unlikely places like Ukraine and Spain [1]. Thus the question of single domestication site or multiple and independent domestication sites is always debatable. Among the different jungle fowls, red jungle fowl i.e. *Gallus gallus* (*G.g.*) is believed to be the sole progenitor of domestic fowl. There are five subspecies of red jungle fowl inhabiting the Indian sub-continent eastwards across Myanmar, South China, Indonesia to Java. Among these five, in India, two subspecies namely *G.g. murghi* (Indian red Jungle Fowl) and *G.g. spadicus* (Bermese red jungle fowl) are found. While the former is distributed in the north and central part of India, extending eastwards to Orissa and West Bengal, the later is confined to the Northeastern parts of India. Some studies were made to establish phylogenetic relationship between domestic fowl, different red jungle fowl subspecies and other jungle fowls. A strong possibility of a single domestication event being the *G.g. gallus*, a major or sole contributor [3]. This theory was further substantiated, who suggested that *G.g. gallus* is the real matriarchic origin of all the domestic poultry [4]. They also found no discernible differences among *G.g. spadicus* and *G.g. gallus*, while *G.g. bankiva* showed differences with both. The genetic and phylogenetic relationships among these species [5]. It provides a framework for genetic studies in wild jungle fowls and native and domestic chicken breeds. These studies have excluded the two other subspecies of red jungle fowl i.e. *G.g. murghi* and *G.g. jabouillei*. In view of strong evidences of domestication of chicken in Indus valley, it may be interesting to study the genetic relatedness between Indian red jungle fowl and other red jungle fowl subspecies including *G.g. domesticus* and other jungle fowls. Studied the genetic diversity of native fowls in Laos by analyzing a mitochondrial DNA (mtDNA) sequence polymorphism, multiple maternal lineages were involved in the origin of domestic chicken in Laos [6]. Moreover, there appear to be at least two maternal lineages, one from China and the other from the Southeast Asian continent. Hence in present study an attempt has been made to study the nucleotide sequence variation in most variable region of mitochondrial D loop region to establish phylogenetic relationship among them.

MATERIAL AND METHODS

The primers were designed to amplify the D loop region. The forward primer was taken from tRNA-Glu (5'-AGG ACT ACG GCT TGA AAA GC-3'), while the reverse primer was taken from tRNA-Phe (5'-CAT CTT GGC ATC TTC AGT GCC A-3'). PCR reactions were set up in 25 µl reaction volume containing 2.5 µl of 10 X Assay buffer (100 mM Tris- HC1, pH 9.0, 15 mM $MgCl_2$, 500mM KC1 and 0.1% gelatin), 200 µM of dNTP mix, 10 pm of forward and reverse primer, 1U Taq DNA polymerase, 50 ng of genomic DNA and autoclaved milliQ water to make up the volume. The amplification was carried out in an i-cycler (Biorad)). Protocol for PCR reaction consisted of an initial denaturation at 94°C for 5 min. followed by 35 cycles of PCR, each cycle consisting of 1 min at 94°C, 1 min at 55 °C and 2 min at 72°C, and followed by a final extension step of 10 min at 72 °C. The PCR products were resolved on 1.6 % agarose gel in 1x TBE. Electrophoresis was done at 90 volts for 10 minutes, then at 50 volts for 2 hour. Gel was viewed under a UV light. The amplified product was purified, cloned in pTZ57R/T vector, MBI Fermentas and sequenced using automated sequencer using Sanger's dideoxy chain termination method. The related sequences (Table 1) were obtained from Genebank (*www.ncbi.nlm.nih.gov*). The sequences were edited by using GENETOOL software to get comparable sequences. Subsequently, the sequences were aligned using CLUSTALW [7]. Website (*http://www.cbi.ac.uk/clustalw*). Jukes-Cantor genetic distances were estimated

using the computer program Molecular Evolutionary Genetic Analysis (MEGA Version 4.0). Jukes-Cantor estimates were used because all mitochondrial sequences were not much divergent (< 6 % divergence from raw counts) and no strong transition bias was evident. The Molecular Evolutionary Genetic Analysis (MEGA Version 4.0) software was used to estimate nucleotide as well as amino acid variability. The genetic distances were estimated as Kimura 2-parameter distances using MEGA software. Phylogenetic trees were constructed with neighbour joining (NJ) procedure using MEGA Version 4.0. Support of the clusters was evaluated by bootstrap, as percentage recurrence of clusters based on 100 bootstrapped replications with MEGA Version 4.0.

RESULTS

The complete D loop in Indian red jungle fowl was 1235 bp and among them, the Domain I of the D loop region was found to be most polymorphic (Fig 1). Hence the nucleotide sequence variability in this region was used to establish the phylogenic relationship of Indian red jungle fowl (*G. gallus murghi*) with the other three subspecies of Red Jungle Fowl. A total of 13 nucleotide sequences (Table 1) of first 400 nucleotide of mt D loop region from different subspecies of Red Jungle Fowl, including those from Indian Red Jungle Fowl (*G.g. murgh*i) were used to study the nucleotide variability in this region. Out of 400 sites about 12 % were polymorphic between the *Gallus gallus* subspecies. Among the 45 nucleotide substitutions, 60 % were transitions (22 T/C and 5 G/A), while 18 were transversions (4 T/G, 10 Y/A, 1 C/G and 3 C/A).

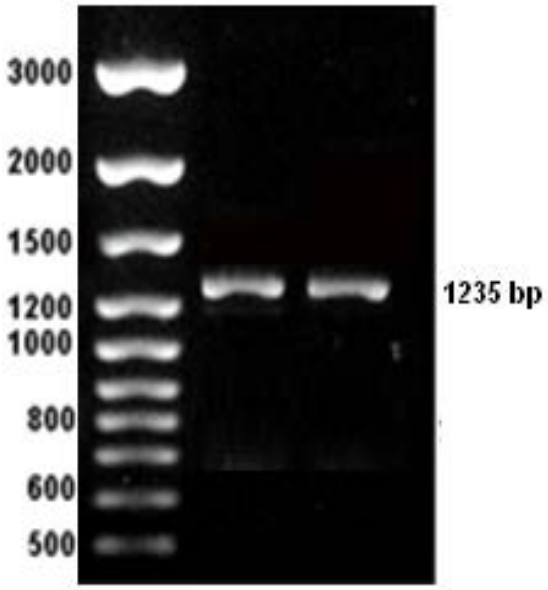

Fig 1. Amplification of 1235 bp fragment in RJF (Lane 1-3 . M 100 bp ladder plus

Table 1. Localities of the different samples from subspecies of Red Jungle Fowl

Name of Animal	Code	Place of Collection	Reference
G. gallus murghi	Indian RJF	Pilibhit, UP, India	Present work
G. gallus bankiva	GG_bankiva15	Singaraja, Bali, Indonesia	Fumihito et al. (1996)
	GG_bankiva18	West Java, Indonesia	Fumihito et al. (1996)
	GG_bankiva19	Lanpung, East Sumatra, Indonesia	Fumihito et al. (1996)
G. gallus gallus	GG_gallus11	Tama Zoological Garden, Tokyo, Japan	Fumihito et al. (1996)
	GG_gallus39	Palembang, East Sumatra, Indonesia	Fumihito et al. (1996)
	GG_gallus58	Palembang, East Sumatra, Indonesia	Fumihito et al. (1996)
	GG_gallus8	Dept. of Forestry, Thailand	Fumihito et al. (1996)
	GG_gallus10	Dept. of Forestry, Thailand	Fumihito et al. (1996)
	GG_gallus3322	Manila, Philippines	Nishibori et al., 2005
G. gallus spadicus	GG-spadiceus3	Dept. of Forestry, Thailand	Fumihito et al. (1996)
	GG-spadiceus4	Dept. of Forestry, Thailand	Fumihito et al. (1996)
	GG-spadiceus5	Dept. of Forestry, Thailand	Fumihito et al. (1996)

Among red jungle fowl subspecies, showed comparatively higher genetic distances (0.008 to 0.036) with each other in *G.g. gallus* birds as compared to the *G.g. spadiceus* birds (0.003-0.008) and *G.g. bankiva* birds (0.013-0.015). It suggested comparatively more within-population variability in *G.g. gallus* as compared to other two subspecies. While Indian RJF (*G.g. murghi*) showed very low genetic distances (0.008-0.013) with some *G.g. gallus* birds (*GG_gallus*8, *GG_gallus*10 and *GG_gallus3322*), with other *G.g. gallus* samples i.e. *GG_gallus*11, *GG_gallus*39 and *GG-gallus*58, the genetic distances were comparatively higher (0.018-0.034). With other two RJF subspecies, *G.g. murghi* showed comparatively high genetic distance i.e. 031-0.034 with *G.g. spadiceus* and from 0.061-0.066 with *G.g. bankiva* (Table 2).
The species *Gallus gallus* is composed of four sub-species i.e. *G.g. murghi*, *G.g. gallus, G.g. spadiceus* and *G.g. banikva*. The phylogenetic tree showed that *G.g. banikva* is well separated from the other three subspecies as it made a separate cluster. *G.g. gallus* makes two separate clusters i.e. one those from Thailand (*GG_gallus*8, *GG_gallus*10 and *GG_gallus*3322, GG_gallus11) and other those from Indonesia (*GG_gallus*39 and *GG_gallus*58). The *G.g. murghi* falls in one sub-cluster along with the *G.g. gallus* from Thailand. In same cluster, all the *G.g. spadiceus* made one separate sub-cluster. Based on nucleotide divergence of 480 bp of D loop region, showed that domestic fowls including Indonesian races belong to the same cluster as a continental population of *G. g. gallus* and *G. g. spadiceus* sampled from Thailand and its adjacent areas [4]. On the other hand, three specimens of *G. g. gallus* from South Sumatra form a separate cluster. Presence of Indian RJF in the first cluster showed its

closeness to the other two Gallus gallus subspecies i.e. *G.G. gallus* and *G.g. spadiceus*, which are supposed to have contribution towards evolution of domestic fowl. We found that the genetic divergence between these types of chickens was very low and the phylogenetic tree revealed that each strain of native chicken belonged to each other with the same cluster [8; 9]. In addition, each strain has its own cluster in some individuals (Fig 2).

Table 2. Genetic distances (Jukes-Cantor) between different *Gallus gallus* subspecies based on first 400 nucleotide sequence of D loop region.

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
[2	0.061											
[3]	0.064	0.013										
[4]	0.066	0.015	0.013									
[5]	0.018	0.053	0.055	0.064								
[6]	0.029	0.047	0.050	0.058	0.021							
[7]	0.034	0.058	0.061	0.069	0.031	0.010						
[8]	0.013	0.058	0.061	0.064	0.015	0.026	0.036					
[9]	0.008	0.064	0.061	0.064	0.015	0.026	0.031	0.010				
[10]	0.010	0.055	0.064	0.061	0.013	0.023	0.034	0.008	0.008			
[11]	0.031	0.078	0.075	0.078	0.034	0.034	0.045	0.029	0.029	0.026		
[12]	0.034	0.081	0.078	0.081	0.036	0.036	0.047	0.031	0.031	0.029	0.003	
[13]	0.036	0.083	0.081	0.083	0.039	0.039	0.050	0.034	0.034	0.031	0.005	0.008

[1] Indian-rjf [2] GG_bankiva5 [3] GG_bankival8 [4] GG_bankiva19 [5]gallus11
[6] GG_gallus39 [7] G_gallus58 [8] GG_gallus8 [9] GG_gallus10 [10]GG_gallus3322
[11] GG_spadiceus3 [12] GG_spadiceus4 [13] GG_spadiceus

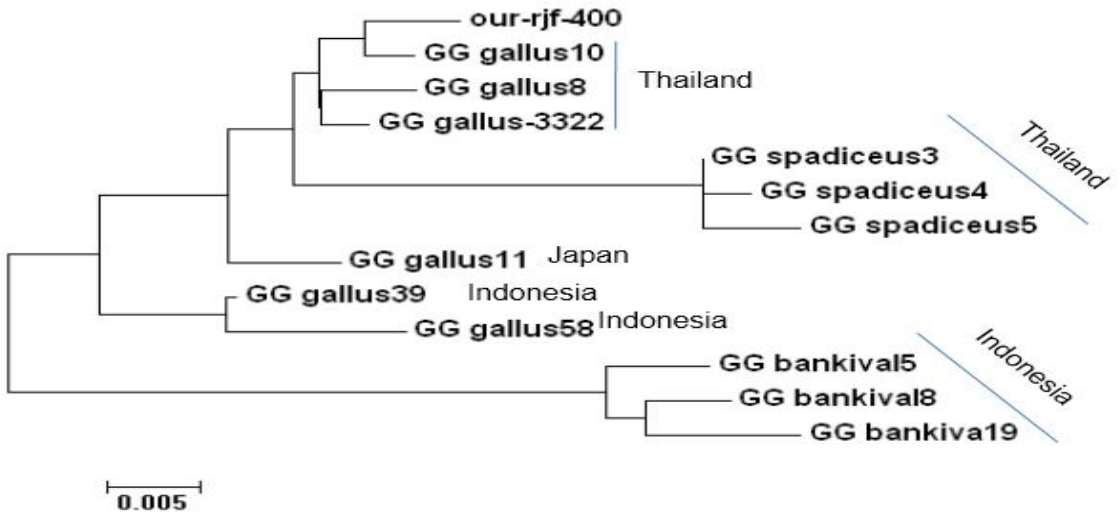

Fig 2. Phylogenetic tree constructed by NJ method based on the sequence of first 400 nucleotides of the D loop region in Indian red jungle fowl and other subspecies.

DISCUSSION

Since most of the sequences of mt D loop region from three species of RJF [4], hence the trend for phylogenetic relationship among them were similar as described by them. But we were more interested to know the phylogenetic relationship of Indian RJF with these RJF subspecies. Our results placed the Indian RJF very close to the *G.g. gallus* from Thailand (Continental population), which were more nearer to *G.g.* spadiceus. Very few reports are available on phylogenetic relationship of Indian RJF with other RJF subspecies. Studied the genetic divergence between Indian Red Jungle Fowl (*G.g. murghi*), *Gallus gallus* subspecies (*G.g. spadicus*, *G.g. gallus* and *G.g. bankiva*) including *G.g. domesticus* (domestic fowl) and three other *Gallus* species (*G. varius*, *G. lafayetei*, *G. sonneratii*) in two ribosomal genes (12S rRNA and 16S rRNA) and found that *G.g. murghi* was more close to *Gallus gallus* subspecies in comparison to other jungle fowls [10], however between Indian RJF and other RJF subspecies, the divergence was very low. Very recently, Suggested the evidence for domestication of Indian birds from *G.g. spadiceus* and *G.g. gallus* as well as from *G.g. murghi*, corroborating multiple domestication of Indian and other domestic chicken [11]. The proposed SNP panel can effectively be used to characterize the four Thai indigenous chickens, these indigenous chicken breeds were more closely related to red jungle fowls than those of the commercial breeds [12; 13]. Neighbor-joining tree using genetic distance revealed that the native chickens from two countries were genetically close to each other and remote from Red and Green jungle fowls of Java Island [12]. The Phylogenetic analysis showed the close genetic relationship within and between the populations of each country and molecular information on genetic diversity revealed may be useful in developing genetic improvement and conservation strategies to better utilize of precious genetic reserve [14;15]. The genetic information from this study is the initial investigation using these populations in Thailand, Indonesia, Japan and India which may be useful in developing future strategies for conservation and improvement of valuable genetic resource.

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