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Genetic Diversity Studies of Red Junglefowl Across its Distribution Range in Northern India

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ABSTRACT

Genetic diversity of Red Junglefowl (*Gallus gallus*) in five range states of northern India was explored to make conservation action plan for genetically endangered bird species. A total of 111 Red Junglefowl samples were collected and genotyped with 15 polymorphic microsatellite loci. Altogether 173 alleles were found with mean (±SE) allelic number of 11.53±6.96. Loci LEI0234 yielded the highest number of alleles (Na = 32) while loci LEI0166, MCW0222 and MCW0098 yielded the lowest number of alleles (Na = 5) in the population. Out of 15 microsatellite loci used, 14 loci showed PIC value higher than 0.5 and considered informative for population genetic studies. The observed heterozygosity (Ho) in the population ranged between 0.378 and 0.637, with mean (±SE) of 0.391±0.134. Hardy-Weinberg equilibrium (HWE) estimations were conducted and all the 15 loci were found to follow the equation ($p>0.05$). No evidence for linkage disequilibrium was observed among pair of loci. Genetic bottleneck hypotheses were also tested, suggesting that RJF has not experienced a genetic bottleneck in the recent past. Persent study revealed the considerable genetic variation in this population, which is an important factor for genetic resources conservation, evaluation and for conservation breeding.

Key words: Red junglefowl, *Gallus gallus*, microsatellite markers, genotyping, genetic diversity, genetic bottleneck

INTRODUCTION

The wild Red Junglefowl (*Gallus gallus*) (RJF) is the wild ancestor of all domestic chicken in the world and is one of the most important species to mankind due to the economic and cultural significance to human civilization (Fumihito *et al*., 1996). The present day multi-billion dollar poultry industry is based on the wild RJF and will depend on it in future as well. India is the fifth largest egg producer with over 40 billion eggs and over 650 million broilers (www.fao.org). The evolution of chicken can be conceived as a three steps processes-evolution and speciation of wild ancestor; domestication of wild species, and diversification into numerous varieties under artificial selection by humans. Exotic high yielding breeds have replaced our native breeds. The populations of domestic animals and their wild ancestors provide a valuable source of genetic diversity that may be exploited to develop animal models for quantitative traits of biological and medicinal interest. Conservation of genetically pure wild forms or their representatives have great potential to make

significant contribution to the study of some economically important genetic traits of domestic forms. Unlike other domestic species, where the ancestor from which the present day animals evolved do not exist, the domestic chicken offers a unique system to compare the effect of natural selection and therefore it has immense value. Hanotte and Jianlin (2005) stated that the reservoir of genetic diversity the putative wild ancestors' are now either extinct or low in numbers and threatened by extinction. Although RJF is considered abundant both in captivity and in wild but due to hybridization with feral or domestic chicken, the wild RJF is believed to be threatened with genetic endangerment throughout its distribution range (Peterson and Brisbin, 1998). Habitat loss, fragmentation, degradation and poaching have also threatened the wild RJF populations in India. Recent studies (Brisbin and Peterson, 2007; Kanginakudru *et al.*, 2008) suggest that phenotypic characters may have little, if any, utility in characterising RJF stock as to their genetic purity. To approach these questions of genetic purity, however apart from phenotypic markers, additional tools drawn from molecular genetics have yet to be applied. Currently, microsatellites are widely used since they are numerous, randomly distributed in the genome, highly polymorphic and show co-dominant inheritance (Karaca *et al.*, 1999). Many microsatellites have recently become available in chicken and have been mapped in reference populations (Crooijmans *et al.*, 1996a; Groenen *et al.*, 2000). These markers provide a powerful tool for quantitative trait loci (QTL) research and have also been successfully used to study the genetic relationship between and

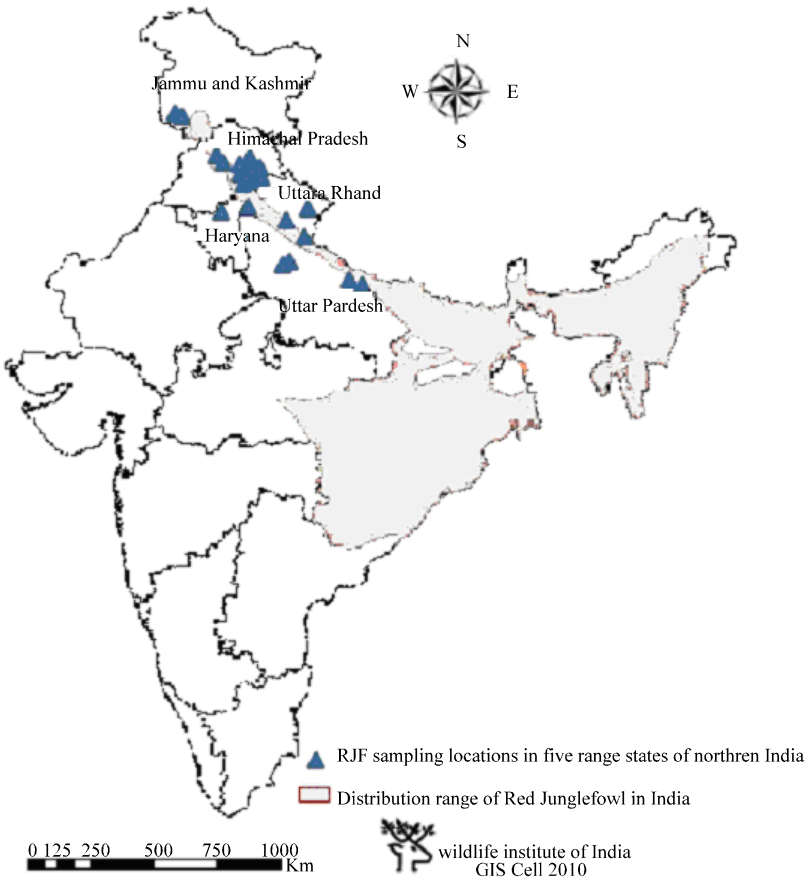

Fig. 1: Map showing Red Junglefowl sampling location in northern India along with its distribution in five different zones in India

within chicken populations (Vanhala *et al.,* 1998; Ponsuksill *et al.,* 1999; Zhou and Lamont, 1999; Romanov and Weigend, 2001 Rosenberg *et al.,* 2001). Reliable information on allele frequencies was obtained from chicken blood or DNA pools using minisatellite markers (Dunnington *et al.,* 1994) as well as microsatellites (Khatib *et al.,* 1994, Crooijmans *et al.,* 1996b, Tadano *et al.*, 2007, Bao *et al.,* 2007, Mukesh *et al.*, 2010a). Some research on origin, phylogeny, genetic diversity and domestication of RJF (Fumihito *et al.*, 1996; Vanhala *et al.*, 1998; Romanov and Weigend, 2001; Niu *et al.*, 2002; Moiseyeva *et al.*, 2003; Hillel *et al.*, 2003; Olowofeso *et al.*, 2005; Ya-Bo *et al.*, 2006; Sharma, 2006; Kanginakudru *et al.*, 2008; Hassen *et al.*, 2009) have been carried out. The RJF is widely distributed and its five subspecies are spread from the Indian subcontinent eastwards across Myanmar, South China, Indonesia to Java (Johnsgard, 1986). In India, two sub-species occur, the type specimen, *Gallus gallus murghii* and *Gallus gallus spadiceus* (Fernandes *et al.*, 2009). While the former is found in the north and central part of India, extending eastwards to Orissa and West Bengal, the latter is confined to the northeastern parts of India. In order to obtain information on the current status and distribution of RJF in India, extensive surveys were carried out in different range states that fall under five regions viz., Northern, Central, Eastern, Southeastern and Northeastern (Fig. 1). The species is present in 205 Districts in 21 range states in India. Of the 255 Protected Areas that occur within the RJF distribution range in India, 190 (31 National Parks and 159 Wildlife Sanctuaries) have reported presence in their areas (Fernandes *et al.*, 2009).

MATERIALS AND METHODS

Live-trapping, sample collection and DNA extraction: In total, 111 RJF samples (16 from wild and 95 from captivity) were collected from five range states of northern India (Table 1). RJF were live-trapped across its distribution range states in northern India, which includes Jammu and Kashmir, Himachal Pradesh, Haryana, Uttarakhand and Uttar Pradesh. We also collected blood/feather samples from captive RJF stock in zoos/pheasantries in these States. In addition, the efficacy of different sampling methods and analytical techniques for g DNA extraction with considering cost and efforts have also been conducted (Mukesh *et al.*, 2010b). Approximately

Table 1: Details of Red Junglefowl samples collected from 5 distribution range states in northern India

RJF Range State of Northern India	Protected Areas	Wild RJF	Captivity Centre/Zoo	Captive RJF
Jammu and Kashmir	Nandani WS	2	--------	0
Himachal Pradesh	Simbalbara WS	2	Chail pheasantry	8
			Renuka pheasantry	12
			Kufri pheasantry	19
			Shimla pheasantry	15
			Gopalpur pheasantry	23
Haryana	--------	0	Morni RJF Breeding Centre	9
Uttarakhand	Rajaji NP	2	Nanital Zoo	2
	CTR-Corbett Tiger Reserve		1	
	WII Campus	4		
	Terai East FD	1		
Uttar Pradesh	Dudhwa	2	Kanpur Zoo	1
	Katerniaghat	1	Lucknow Zoo	6
	Kishanpur WS	1		
Total		16		95

500 μL blood was drawn from live-trapped bird and stored in DNA zol BD as well as on FTA cards (Mackey *et al.*, 1996, 1998). Freshly pulled primary feathers were preserved in 70% alcohol (www.gallus.forestry.uga.edu/ggg/protocols/pdf/feather.pdf) while moulted feathers and hatched egg shell were stored in an envelopes/zip lock bag (www.absc.usgs.gov/). Genomic DNA was extracted from blood following DNA zol BD based protocol while DNA was extracted from tissue, feathers and egg shell membrane using Qiagen DNeasy tissue kit, following the manufacturer's instructions. This present study is the part of a national project titled 'Conservation of Red Junglefowl in India that is being carried out at the Wildlife Institute of India to develop an action plan for conserving the wild ancestor in its natural habitat. The project was initiated in September 2006 and will conclude by February 2010. Samples are being collected from its potential distribution range in 21 states of India.

Microsatellite genotyping: A set of 15 polymorphic microsatellite markers, developed for chicken and earlier used in the (AVIANDIV *et al.*, 2000) project were used in the study (Table 2) (www.aviandiv.tzv.fal.de/primer_table.html). The PCRs were carried out in 10 μL reaction volume in an Applied biosystem thermal cycler (2700 and 2720), containing 1 X PCR Buffer (50 mM KCl, 10 mM Tris-HCl), 1.5 mM $MgCl_2$, 200 μM of each d-NTP, 1.25 μg BSA, 4 p-mole of each primer, 0.5 unit of Taq DNA polymerase, 50 to 80 ng of gDNA. The amplification conditions were: 2 min initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at specific temperature (Table 2) for 45 sec, and extension at 72°C for 2 min with a final extension at 72°C for 15 min. 5 μL of PCR products were mixed with 1 μL of loading buffer and then loaded onto a 2% agarose gel containing ethidium bromide and visualized over UV light to detect amplification. PCR products were pooled and denatured at 95°C for 5 min and microsatellite genotyping was carried out using ABI 3130 Genetic Analyser (Applied Biosystem) with GeneScan-500 (-250) LIZ as the internal lane size standard. Data were collected and analysed using GeneMapper Software (Version 3.7, Applied Biosystem) (Estes-Zumpf *et al.*, 2010).

Table 2: Description of Microsatellite Loci used in the study

		Primer sequence (5' -> 3')			Annealing
Loci	Chromosome	Forward	Reverse	Allele range	temperature T_A (°C)
ADL0268	1	CTCCACCCCTCTCAGAACTA	CAACTTCCCATCTACCTACT	102-116	60
LEI0166	3	CTCCTGCCCTTAGCTACGCA	TATCCCCTGGCTGGGAGTTT	354-370	60
MCW0037	3	ACCGGTGCCATCAATTACCTATTA	GAAAGCTCACATGACACTGCGAAA	154-160	64
ADL0112	10	GGCTTAAGCTGACCCATTAT	ATCTCAAATGTAATGCGTGC	120-134	58
MCW0295	4	ATCACTACAGAACACCCTCTC	TATGTATGCACGCAGATATCC	88-106	60
MCW0067	8	GCACTACTGTGTGCTGCAGTTT	GAGATGTAGTTGCCACATTCCGAC	176-186	60
MCW0111	1	GCTCCATGTGAAGTGGTTTA	ATGTCCACTTGTCAATGATG	96-120	60
MCW0034	2	TGCACGCACTTACATACTTAGAGA	TGTCCTTCCAATTACATTCATGGG	212-246	60
MCW0222	3	GCAGTTACATTGAAATGATTCC	TTCTCAAAACACCTAGAAGAC	220-226	60
MCW0081	5	GTTGCTGAGAGCCTGGTGCAG	CCTGTATGTGGAATTACTTCTC	112-136	60
MCW0330	17	TGGACCTCATCAGTCTGACAG	AATGTTCTCATAGAGTTCCTGC	256-300	60
LEI0234	2	ATGCATCAGATTGGTATTCAA	CGTGGCTGTGAACAAATATG	216-364	60
MCW0098	4	GGCTGCTTTGTGCTCTTCTCG	CGATGGTCGTAATTCTCACGT	261-265	60
MCW0078	5	CCACACGGAGAGGAGAAGGTCT	TAGCATATGAGTGTACTGAGCTTC	135-147	60
MCW0165	23	CAGACATGCATGCCCAGATGA	GATCCAGTCCTGCAGGCTGC	114-118	60

Genetic diversity analysis: Genetic diversity estimates *i.e.,* Observed and expected heterozygosity, observed and effective number of alleles were carried out after Nei (1972, 1973) using POPGENE software Francis *et al.*, 1999). Using allelic frequencies, polymorphic information content (PIC), a measure of marker's informativeness, was calculated with the Cervus (ver. 3.0) computer program (Kalinowski *et al.,* 2007). Deviation from Hardy-Weinberg equilibrium was tested using the exact test of POPGENE (Francis *et al.*, 1999). Heterogeneity of deviations from Hardy-Weinberg equilibrium among the microsatellite loci was investigated by considering the deviations as correlation coefficients and tested accordingly (Barker *et al.*, 2001). Heterozygote deficiencies were estimated as FIS = (Ho- He)/He, where Ho and He are the observed and expected frequency of heterozygotes, respectively. Linkage disequilibrium (LD) test between pairs of loci was performed in FSTAT version 2.9.3 (Goudet, 1995). Evidence of a recent genetic bottleneck was tested using the program BOTTLENECK (Piry *et al.*, 1999)

RESULTS AND DISCUSSION

Polymorphism of markers and genetic diversity statistics: In this study, RJF population of northern India showed enough genetic polymorphism. All 111 RJF samples were successfully amplified and 173 distinct alleles were distinguished over the 15-microsatellite loci used. Linkage disequilibrium was not detected between the investigated loci. Therefore all the loci were retained for the further analysis. Genetic diversity measures of RJF population are presented in Table 3. The number of observed alleles were ranged from 5 for loci LEI0166, MCW0222, MCW0098 and 32 for locus LEI0234 while the overall mean number of alleles per locus was reported 11.53 (±6.9 SE). The effective number of alleles were ranged from 2.31 for locus MCW0098 to 10.05 for locus LEI0234 with mean (±SE) of 4.67±2.10 (Table 3). The observed number of alleles for all the 15 loci exceeded the effective number of alleles. All the microsatellite loci showed PIC values higher than 0.5 except MCW0098 (PIC-0.472) which is normally considered as informative in population-genetic analyses (Botstein *et al.*, 1980). The mean PIC in the present study was 0.7119. Mean (±SE) observed heterozygosity over the 15 loci was 0.391±0.134, which was lower than the expected heterozygosity. The expected heterozygosity within RJF population of northern India ranged from 0.570 (MCW0098) to 0.905 (LEI0234) with overall mean (±SE) of 0.755±0.090. Thus RJF population of Northern India gave the impression of high-quality reservoir of genetic variation which also corroborates with our own study carried out in state of Himachal Pradesh with captive samples (Mukesh *et al.*, 2010a). Our within-population inbreeding estimate (FIS) was significantly positive, as derived from table-wide randomizations ($p<0.05$). The FIS estimates ranged between 0.230 for locus MCW0081 to 0.755 for locus MCW0037. The mean FIS estimate was 0.478. Heterozygosity, however, is not reduced proportionally, because rare alleles contribute little to heterozygosity. Recent genetic bottleneck was tested using the statistical test based on the difference between allelic diversity and heterozygosity (Piry *et al.*, 1999).The allele frequency spectrum was visualized by the qualitative graphical method as described by Cornuet and Luikart (1996) (Fig. 2). The microsatellite alleles were arranged into 10 frequency classes, which make sure whether the distribution followed the normal L-shaped form, where alleles with low frequencies (0.01-0.1) are the most abundant. The observed allelic distribution revealed that the population did not encounter a genetic bottleneck in the recent past. The significant level of genetic variability in RJF population of northern India, notwithstanding its diminished population size, is indicative of a valuable reservoir of genetic diversity in this population.

Table 3: Genetic Diversity statistics of Red Junglefowl in northern India

Locus	Number of alleles (n_a)	Effective number of alleles ($ne^{\S}$)	Shannon's information index ($I^{\S}$)	Polymorphic information content (PIC)	Observed heterozygosity (H_o)	Expected heterozygosity (H_e*)	Nei*	eterozygote deficiency (F_{Is})
ADL0268	17	6.9114	2.2963	0.844	0.5825	0.8595	0.8553	0.3189
LEI0166	5	3.1169	1.2637	0.621	0.2754	0.6841	0.6792	0.5946
MCW0037	8	2.99	1.2782	0.609	0.1625	0.6697	0.6655	0.7558
ADL0112	8	3.8697	1.5971	0.706	0.4568	0.7462	0.7416	0.384
MCW0295	16	7.5359	2.2232	0.854	0.3429	0.8715	0.8673	0.6047
MCW0067	16	3.1179	1.5701	0.631	0.2667	0.6825	0.6793	0.6074
MCW0111	7	3.9739	1.4826	0.706	0.5132	0.7533	0.7484	0.3143
MCW0034	12	4.7858	1.86	0.764	0.4512	0.7959	0.791	0.4296
MCW0222	5	3.0442	1.1666	0.602	0.382	0.6753	0.6715	0.4311
MCW0081	13	5.8026	1.9358	0.805	0.6373	0.8317	0.8277	0.2301
MCW0330	8	3.8699	1.5469	0.7	0.378	0.7461	0.7416	0.4902
LEI0234	32	10.0575	2.736	0.893	0.404	0.9051	0.9006	0.5514
MCW0098	5	2.3102	0.9475	0.472	0.1728	0.5707	0.5671	0.6952
MCW0078	10	5.0425	1.7712	0.773	0.4158	0.8057	0.8017	0.4813
MCW0165	11	3.7643	1.6072	0.7	0.4348	0.7384	0.7343	0.4079
Mean	11.5333	4.6795	1.6855	0.7119	0.3917	0.7557	0.7515	0.4787
s.e.‡	6.968	2.1067	0.4714		0.1347	0.0908	0.0906	

§ne: effective number of alleles (Kimura and Crow 1964), I: Shannon's information index (Lewontin 1972); * :H_e :Expected heterozygosity was cmputed using Levene (1949) and Nei (1973) epected heterozygosity, ‡ s.e.:- Standard error

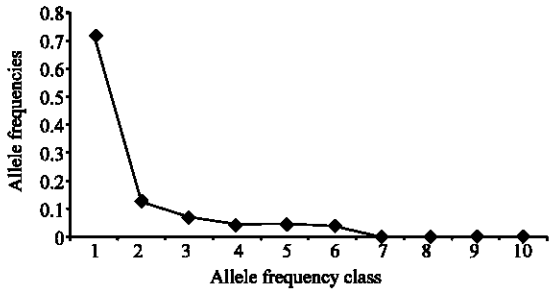

Fig. 2: Normal 'L-shaped mode-shift' graph showing lack of recent genetic bottleneck in Red Junglefowl population in northern India

CONCLUSION

The high genetic diversity in this population is important for genetic resources conservation and utilization. The information from the DNA markers together with phenotypic performance and population history provides reliable guidelines that can be used in developing practical strategies for conservation purposes and for breeding programs. High-priority action plan has to be initiated and the villagers especially tribal should be educated for comprehensive safeguarding of wild RJF by not selectively removing males from wild populations for upgrading their domestic breeds which may further weaken the diversity levels through limitation of wild mating males.

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