5.0 Conservation of Red Junglefowl (*Gallus gallus*) in India – Towards mapping Abundance and Genetic Diversity

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Introduction

The Red Junglefowl (*Gallus gallus*) [RJF] is one of the most important species to mankind due to the economic and cultural significance to human civilization. According to Liu *et al* (2006) there are distinct distribution patterns and expansion signatures suggesting that different clades of chicken might have originated from different regions, which also support the theory of multiple origins in South and Southeast Asia. In other words, the present day multi-billion dollar poultry industry is based on the wild RJF and may have to depend on it in the future as well. India is the fifth largest egg producer with over 40 billion eggs and over 650 million broilers (www.fao.org). Andersson *et al*. (1994) have stated that '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 medical 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 the domestic form (Brisbin 2002).

The RJF has widespread distribution and in its five sub-species are spread from the Indian sub-continent eastwards across Myanmar, South China, Indonesia to Java (Johnsgard 1986). In India, two sub-species of RJF occur, the type specimen, *Gallus gallus murghii* and *Gallus gallus spadiceus* (Ali and Ripley 1983). 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 North-eastern parts of India. Recently, fears have been expressed that the wild RJF populations may be genetically contaminated leading to an inference that there may not be any pure RJF populations in the wild (Peterson and Brisbin 1998). They may have hybridised with feral and or domestic stock, especially near the villages causing introgression of domestic genes into the wild populations. The skins that were examined by Peterson and Brisbin (2001) showed lack of phenotypic traits, which characterise true wild RJF as described by Morejohn 1968.

Further, the pattern of genetic contamination as suggested by Peterson and Brisbin (1998) in wild RJF is also contentious. Irrespective of the apparent sampling inadequacy, the threat of hybridisation to the RJF in India is real and needs to be addressed urgently. Keeping this in view, a collaborative study was initiated in mid 2006 to provide more information on the status and distribution of RJF in India and ways in which to safeguard the genetically pure wild populations. This paper presents the preleminary findigs of the ongoing study for hte period upto September 2007.

Materials and Methods

Assessing the status and distribution of RJF in India

In order to asses the distribution and status of RJF in India the historical distribution range was divided into five different zones *viz*., North (Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Haryana, Punjab and Uttar Pradesh), Central (Madhya Pradesh and Chhattisgarh), Eastern (Bihar, Jharkhand, West Bengal), Southeast (Orissa and Andhra Pradesh) and Northeastern (Sikkim, Assam, Arunachal Pradesh, Nagaland, Mizoram, Manipur, Tripura and Meghalaya). The 'presence / absence' distribution was assessed based on secondary information from literature, questionnaire surveys, reliable personal communication and field surveys. *Ad libitum* surveys using the encounter rate method were carried out in different habitats and areas to obtain abundance estimates of population (Bibby *et al*. 1992). A minimum of two trails was selected, which was sampled atleast thrice. The encounter rates were pooled together for the different forest types.

Identification of pure RJF populations by molecular genetic studies

A composite set of traits were complied with the help of Morejohn (1968), Johnsgard (1986) Peterson and Brisbin (1998) and Corder (*pers comm*) for identifying the pure wild RJF from that of introgressed hybrids. The traits used were yellowish colour hackle, slender smooth thin blackish legs, white patch at the upper tail coverts, elongated sickle shape central tail feathers, horizontal tail carriage in males while the RJF hen in comparison to a chicken, is slender; smaller in size and has no width across its shoulders, has vestigial or no crest and the tarsus is slender smooth, thin and blackish. In males during the post breeding season it undergoes moulting (eclipse plumage). There were 15 cases where the birds were trapped and measured, measurements of tarsus, bill and comb were noted with the help of a digital vernier calliper. There were some other traits such as semi-transparent thin skin and no protest while handling that was recorded when the bird was in hand.

Sampling

A total of 70 samples of RJF and 43 samples of different breeds of domestic chicken were collected from various parts of India. Out of 70 RJF samples, 32 samples were from wild and 38 samples from captivity. Blood was collected from wing vein of trapped individuals using DNA zol BD and on FTA cards (Mackey *et al* 1997). Freshly pulled primary feathers were directly preserved in absolute alcohol while moulted feathers and hatched egg shell were collected and stored in a zip lock bag for dry preservation.

DNA Extraction from whole blood, tissue, feather and egg shell membrane

Genomic DNA was extracted from blood following DNA zol BD based protocol Mackey *et. al* (1996) (U.S. Patent No.5, 945,515). For tissue, feathers and egg shell membrane DNA was extracted using Qiagen DNeasy tissue kit. DNA was quantified with UV spectrophotometer and concentration was adjusted approx. 50-80 ng/ìl with TE buffer.

Microsatellite markers

Highly polymorphic microsatellite markers were identified using chicken genome database (www.thearkdb.org) for genotyping of RJF and chicken breeds.

Optimisation of cycling condition and amplification of microsatellite loci

For initial standardisation and optimisation of cycling conditions, four fluorescent labelled primers were used. Reported conditions for the amplification of microsatellite loci were tested and then accordingly modified to get optimised results. PCR amplification was performed in a 10µl reaction volume. Each reaction consists 1X PCR Buffer (50 mM Kcl, 10 mM Tris-Hcl), 1.5 mM $Mgcl_2$, 200 µM of each d-NTPs, 25X BSA, 10 p-mole of each primer (forward and reverse), 0.5 unit of Taq DNA polymerase, 50 to 80 ng of genomic DNA. Protocol for PCR reaction was comprised of an initial denaturation at 94^0C for 2 min, followed by 35 cycles of denaturation at 94^0C for 45 s, primer annealing for 45 s at 55^0C, primer extension for 1 min at 72^0C and a final extension of 10 min at 72^0C. About 5µl of PCR product was resolved on 3.0 % Agarose, and 100 bp ladder was used as molecular size markers.

Results

Distribution of RJF *Gallus gallus* in India

The distribution of RJF covers 300 Districts in 21 range states of the India (Fig 1). The RJF is now reported to occur in 136 districts in the 21 range states. Information on the presence / absence of RJF from over 50% of the districts in its distribution range is unknown. Of the 255 PAs that occur within the RJF distribution range in India, 149 PAs (26 NPs and 123 WSs) have RJF. Information on the presence / absence of RJF from *ca*. 33% of PAs is unknown. As of March 2007, there were 209 individuals (74 males: 80 females: 55 chicks) of RJF in captivity in the various zoos and pheasantries in the states of Andhra Pradesh, Delhi, Haryana, Himachal Pradesh and Uttar Pradesh.

As there were no population estimates of RJF in the PAs, an attempt was made to obtain abundance estimates for RJF in PAs. Line transect or Trail sampling was used to obtain encounter rates for RJF in the different forest or habitat types found in the RJF distributon range of India. A total of 38 trails covering 358 km were traversed (546 man hours effort) in the seven broad vegetation / habitat types. The encounters rate was pooled together for similar habitats (Table 1). The encounter rate was high during the summer months in the Shivalik region, however, the higher 'Coefficient of Variation' (CV) value reveals that the sampling size was insufficient.

Morphological traits

In total 246 RJF (126 males and 120 females) were sighted during the field surveys and 17 males and 16 females in captivity from which trait characteristics were recorded. All males had presence of the yellowish colour hackle and the slaty black thin tarsus; the ear lobes were white in Orissa while rest of the places had pinkish ear lobes. The tail carriage was difficult to characterise, but the presence of the sickle feather was prominent in all cases. In 50% females, comb was absent, while 20% had

rudimentary comb while in 30% of the cases it was not possible to detect the comb. This was mainly due to cryptic colouration and its behaviour. Eclipse plumage was also observed in RJFs (n= 5). However captured populations that were examined at the Morni Hill pheasantry during the period (July – September) had shown eclipse plumage.

Isolation of genomic DNA from different tissues

The genomic DNA was extracted from blood, tissue, feather follicles and egg shell membrane. A good quality and quantity of genomic DNA was obtained from whole blood following DNA zol BD protocol (Fig 2). DNA was also extractable from tissue, feather follicle and egg shell membrane, but the quantity as well as quality of genomic DNA extracted from feather follicle and egg membrane was much lower in comparison to the DNA extracted from tissue (Fig 3).

Optimisation of PCR assay for microsatellite genotyping

PCR cycling condition was optimised for four primers *viz*. MCW 034, MCW 295, MCW 111 and LEI 192. Initially, annealing temperature was optimised for these four microsatellite markers. Annealing temperature between 55^{0}C to 65^{0}C was tested in a gradient PCR amplification. For MCW 034, annealing temperature of 64.9^{0}C was found optimum, while for MCW 295, annealing temperature of 55^{0}C showed better amplification. Similarly, optimum annealing temperature for MCW 111 and LEI 192 was 64.9^{0}C (Fig 4). The average allele sizes for MCW 034, MCW 295, MCW 111 and LEI 192 were ~ 230 bp, 96 bp, 104 bp and 271 bp and were as per similar to those reported by Sharma 2006. After optimising the annealing temperature, the microsatellite genotyping was done on some RJF samples and these amplified products were resolved on 3 % agarose gel (Fig 5).

Discussion

The present state wise distribution is nearly the same in comparison to the historical distribution of Hume and Marshall 1879, Ali and Ripley 1983 and Madge and McGowan 2002. The distribution range is restricted in certain states due to geographical barriers, environmental elevation gradients and human disturbances. Within central India, the RJF is not reported beyond the west bank of river Pench (R.Jayapal *pers. comm*). Ali and Ripley (1983) demarcate the southern most distribution of RJF to be near Rajahmundry but there were records of RJF in Eturnagaram WS (Nagula, 1997) but the present survey did not record presence of RJF in this sanctuary till summer 2007. For a better understanding of the spatial distribution of RJF, habitat suitability modelling will be developed involving satellite imagery, and ecogeographical variables. From this predictive distribution, the analysed genetic data will be overlaid so as to give different regions with respect to pure and hybrids.

Due to the varied habitat in the distribution range there were constraints in transects repeatability in different seasons hence detection probabilities were not included in the abundance estimates. However we propose to correct for detection probabilities in the different habitats and seasons for the different zones in India by repeating surveys within these same areas that will give an abundance estimate.

In the present study, different samples were used for DNA extraction. The blood and tissue proved to be very good samples for DNA extraction for RJF. Avian blood having nucleated red blood corpuscles (RBCs) is always the sample of choice for DNA extraction. Earlier studies also showed that genomic DNA of good quality as well as quantity can be extracted from frozen and stored chicken blood (Sharma and Apparao, 2000). Extraction of genomic DNA from feather follicle and egg shell membrane is the non-invasive method. The quantity of genomic DNA extracted was quite low with these samples but was enough for PCR amplification.

PCR amplification is influenced by number of factors such as quality and quantity of genomic DNA, concentration of magnesium ions and dNTPs and annealing temperature (Williams *et al*., 1993) hence, the initial standardisation of PCR amplification conditions are often necessary to get optimum amplification. The annealing temperature 55^{0}C was found optimum for MCW 295 while 64.9^{0}C was the optimum annealing temperature for other three microsatellite markers. These amplified products were resolved on 3 % agarose gel and the average allele size observed for these microsatellite markers were in accordance with that reported by Sharma (2006). Since the microsatellite alleles differ in few base pairs with each other at a given locus, the ordinary agarose gel is not sufficient to differentiate the alleles. Hence for better allele differentiation, the amplified product will to be resolved on metaphor agarose or 4-5 % denaturing polyacrylamide gels or on automated DNA sequencer. Sharma (2006) reported polymorphism not only within RJF population, but also between RJF and chicken breeds on 3.5 % metaphor agarose.

Though these preliminary studies showed the presence of polymorphism within RJF population at these four microsatellite loci but further these microsatellite markers as well as other 17 markers will be used to develop the microsatellite profile of RJF population using automated DNA sequencer. These microsatellite allelic profiles will be utilised for estimating the genetic diversity present within the RJF population. Such estimates will be the suggestive of existing genetic variability between the RJF populations from different

regions of the country. Further genetic distance analysis will be undertaken using the microsatellite allelic profile of RJF with that of chicken and used in testing the purity of RJF. Since the introgression of domesticated chicken genes in wild RJF might affect the phenotypic expression of physical traits such as eclipse plumage, hen comb, leg color, horizontal body posture and tail carriage, simpler and a shorter call, therefore, on identifying of pure RJF, we will try to correlate the genetic purity and the physical traits.

A few sites within the distribution range such as Sariyanj (Himachal Pradesh) will be taken up for intensive studies. These sites will try to address issues with respect to the ecology and behaviour (interactions) of wild RJF with the domestic fowl.

Acknowledgements

We thank the Director and Dean, WII, PCCF's, CWLWs, Park Managers, Zoo Curators and field staff in the RJF Range States. Special thanks to Shri Qamar Qureshi and Dr K. Ramesh for reviews and comments on the manuscript. At WII, special thanks to Dr. S.P.Goyal, Dr. K. Sankar, Shri Pratap Singh, Shri Dhananjay Mohan, Dr. Manoj Aggarwal and Dr. R. Jayapal. We wish to acknowledge the researchers of the WII's All India Tiger Monitoring Programme for providing information on the presence/ absence of RJF. We thank all our field assistants.

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Figure 1 : Distribution of Red Junglefowl *Gallus gallus* in India

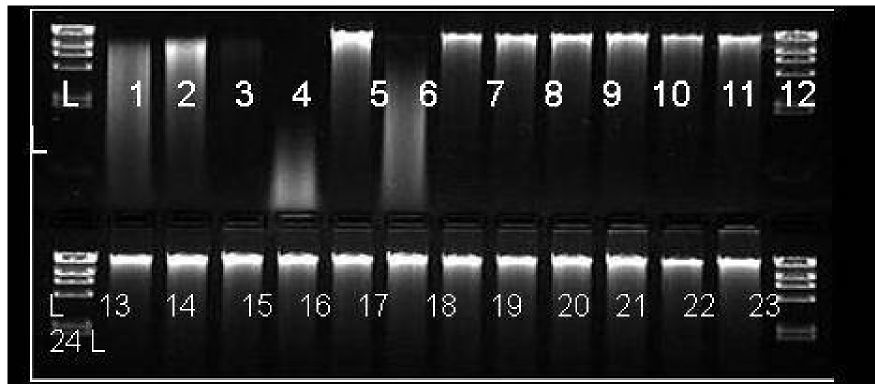

Figure 2. Gel Electrophoresis (0.8% agarose) of extracted DNA, L- 1Kb ladder, Lane- 1 to 12 RJF Samples, Lane- 13 to 24 Domestic Chickens

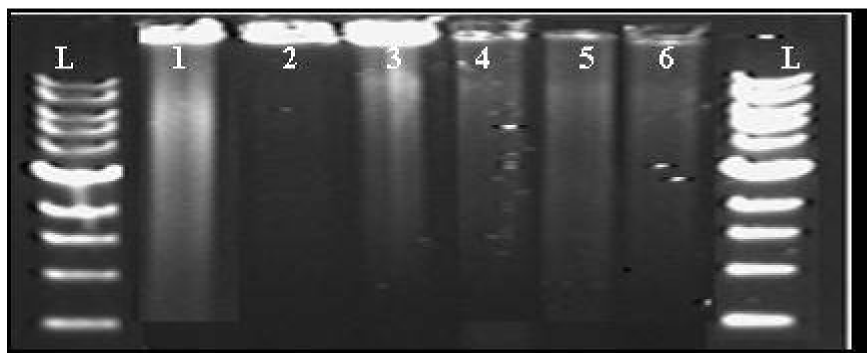

Figure 3. Gel Electrophoresis (0.8% agarose) of extracted DNA, L- 1Kb ladder, Lane- 1, 2,3 DNA from tissue sample, Lane- 4, 5 DNA from feathers and Lane -6 DNA from Egg Shell membrane

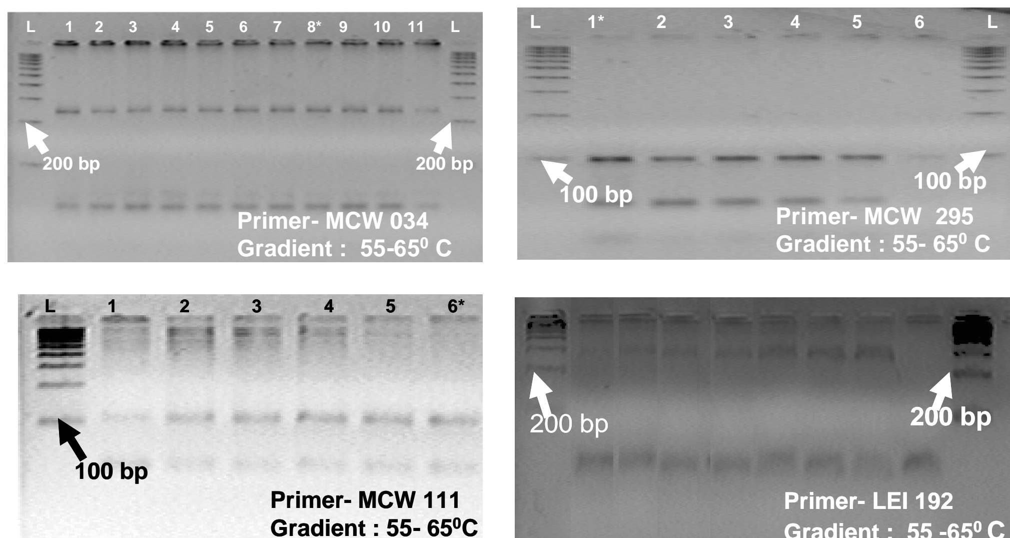

Figure 4. Standardisation of PCR condition for amplification of microsatellite loci
(A) MCW 034, Allele size ~ 230 bp, T_A - 64.9° C, (B) MCW 295, Allele size ~ 96 bp, T_A - 55° C,
(C) MCW 111, Allele size ~ 104bp, T_A - 64.9° C, (D) MCW 192, Allele size ~ 271 bp, T_A - 64.9° C

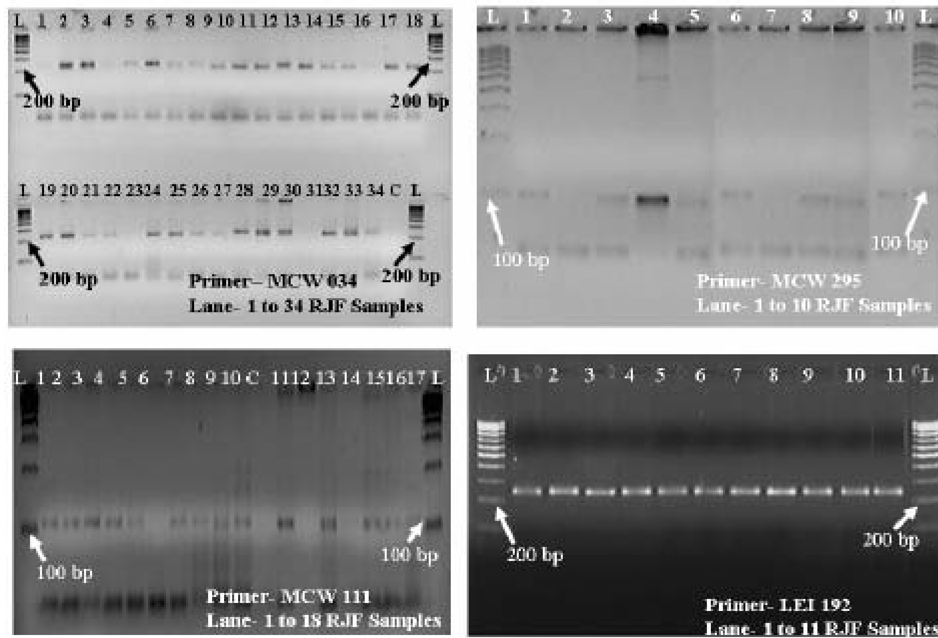

Figure 5. Amplification of microsatellite loci using primer- MCW 034, MCW295, MCW111, LEI 192

Table 1. Encounter rate of RJF in various habitat types of certain Protected Areas.

Forest / Habitat Types	Months	Trails (n)	Total Effort (km)	Mean (no/Km)	Std Error	SampleVariance
Mangroves (Bhittarkanika)	Nov	2 (4)	9.8	1.357	0.295	0.347
Moist mixed forest(parts of Orissa and Udanti WS)	Nov-Dec	6 (10)	46	0.167	0.087	0.076
Dry deciduous (Parts of Andhra Pradesh)	Dec-Jan	11 (16)	103	0.000	0.000	0.000
Moist mixed forest z (Meghalaya and Assam)	Feb-Mar	7 (18)	72	0.466	0.264	0.400
Grassland and Woodland (Assam floodplains)	Mar-Apr	5 (18)	40	1.430	0.202	0.734
Shivaliks (Uttarakhand)	May	4 (12)	19.2	5.056	1.257	18.971
Himalayan Foothills (Himachal Pradesh)	Jun-July	3 (8)	18	0.688	0.298	0.710

(n) = Total number of trails