Conservation of red junglefowl *Gallus gallus* in India

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Abstract The red junglefowl (RJF) is one of the most important species for mankind, due to economic and cultural reasons. Recently, fears have been expressed that the wild RJF may be genetically contaminated, leading to an inference that there may not be any pure RJF left in the wild. In order to assess the distribution of RJF in India, field surveys were carried out and secondary information was collated. Historically, RJF occurred in 270 districts in 21 states across India, but now it is found in 205 districts in the 21 states. Of the 255 Protected Areas (PAs) that occur within the RJF's distributional range in India, 190 PAs (31 National Parks [NPs] and 159 Wildlife Sanctuaries [WSs]) have reported its presence. A composite set of trait characters that are presumed to be indicators of wild RJF was used for characterising RJF in the field. A total of 563 (293 males and 270 females) RJF were characterised of which 7% of birds in the central region had reports of white ear patch. Eclipse plumage was observed in wild and captive birds. Ninety-two RJF samples and twenty five domestic chicken samples were collected and processed for DNA extraction. Thirty highly polymorphic microsatellite markers were utilised for RJF and domestic chicken genotyping. Preliminary studies showed polymorphism within RJF at these microsatellite loci.

Keywords DNA extraction, hybridisation, microsatellite markers, PCR, red junglefowl, traits.

Introduction

The red junglefowl *Gallus gallus* (RJF), the ancestor of the domestic chicken, is one of the most important species for mankind, due to its economic and cultural significance. Liu et al*.,* (2006) suggested that there are distinct distribution patterns and expansion signatures suggesting that different clades may originate from different regions, which support the theory of multiple origins in South and Southeast Asia. The present day, multi-billion dollar poultry industry is based on the RJF and may have to depend on it in the future. Andersson et al. (1994) 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'. Hence conservation of genetically pure wild forms or their representatives have great potential to make a significant contribution to the study of some economically important genetic traits (Brisbin et al., 2002).

The RJF is widely distributed and 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 occur, the type specimen, *Gallus gallus murghii* and *Gallus gallus spadiceus* (Ali & Ripley, 1983). 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 north eastern parts of India. Recently, fears have been expressed that the wild RJF populations may be genetically contaminated with domesticated chickens, leading to an inference that there may not be any pure RJF populations in the wild (Peterson & Brisbin, 1998), causing introgression of domestic genes into wild birds. An analysis of skins by Peterson & Brisbin (1998) showed a lack of phenotypic traits, which characterise true wild RJF, as described by Morejohn (1968).

This study investigated the status of RJF in India and aimed to identify ways to safeguard remaining pure wild birds. In 2006, a collaborative research project on the

conservation of RJF was initiated focussing on, 1) an assessment of the current status and distribution of RJF in India; 2) the identification of pure RJF populations by molecular studies; 3) investigations into social interactions between wild RJF and domestic or feral chicken and 4) the development of a conservation action plan for RJF.

This paper presents the preliminary findings on the current status and distribution of RJF in India, standardisation of DNA extraction protocols for various sample types, optimisation of PCR condition and amplification of seven microsatellite loci in extracted DNA samples. The proposed plan of work is also presented and discussed.

Methods

Assessing the status and distribution of RJF in India

In order to assess the distribution of RJF in India, primary and secondary data was collated. Secondary data was gathered through literature, questionnaire surveys and reliable personal communication. *Ad libitum* surveys using the encounter rate method were carried out to obtain abundance estimates using direct (sightings) or indirect (calls) methods (Bibby et al., 1992). The data (primary and secondary) were digitalized in a Geographical Information System (GIS) using ARCinfo software. A rule-based model was created using forest cover and elevation below 2500 m. A minimum of two transects were selected within represented habitat types, which were traversed a minimum of three times. The encounter rates were pooled for the different forest types.

Identification of pure RJF by molecular genetic studies

A composite set of traits were complied to identify pure wild RJF from introgressed hybrids (Morejohn, 1968; Johnsgard, 1986; Peterson & Brisbin, 1998; Kaul et al., 2004). The traits used were yellow-coloured hackles, slender smooth thin black legs, a white patch on the upper tail coverts, elongated sickle shape central tail feathers and horizontal tail carriage in males. Males moult during the post breeding season and have an eclipse plumage.

Sampling for genetic analysis

Wild RJF were live-trapped using modified foot-hold snares (Ramesh & Kalsi, 2007) with the help of local bird-trappers in different RJF range states in India. The birds were carefully handled during sample collection and released. Samples were also collected from the captive RJF. Blood was collected from the brachial vein of live trapped individuals and stored in DNA zol BD as well as on FTA cards (Mackey et al*.,* 1997). Freshly pulled primary feathers were stored in 70% alcohol, while moulted feathers and hatched egg shells 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. For tissue, feathers and egg shell membrane DNA was extracted using Qiagen DNeasy tissue kit (Qiagen, Germany) according to the manufacturer's protocol with the following alterations: addition of 100 mg/ml DTT solution in the lysis buffer, digestion was performed overnight at 55^0C in a shaking water bath and addition of ice chilled ethanol for better precipitation and DNA was finally recovered in 80-100 µl of elution buffer. DNA was quantified with UV spectrophotometer and concentration was adjusted to approximately 50-80 ng/µl with TE buffer.

Microsatellite markers

A set of thirty highly polymorphic microsatellite markers were utilised for genotyping of RJF and domestic chicken (TABLE 3). (http:// dad.fao.org/en/refer/library /guidelin/marker.pdf)

Optimisation of cycling condition and amplification of microsatellite loci

For initial standardisation, reported conditions were tested first and modified to obtain optimal results. Annealing temperature for each locus was optimised in a gradient PCR and seven microsatellite loci were amplified in extracted DNA samples. PCR amplification was performed in a 10 µl 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 minute, followed by 35 cycles of denaturation at 94^0C for 45 seconds, primer annealing for 45 seconds at 55^0C, primer extension for 1 minute at 72^0C and a final extension of 10 minutes at 72^0C. About 5 µl of PCR product was resolved on 2.0 % Agarose, 100 bp ladder was used as molecular size markers.

TABLE 1 Collection of RJF and domestic chicken samples in different states

State	RJF Samples		Domestic Chicken Samples
	Wild	Captivity	
Jammu and Kashmir	2	-	9
Himachal Pradesh	2	25	1
Uttarakhand	9	2	4
Haryana	-	9	2
Uttar Pradesh	-	16	-
Chhatisgarh	2	-	1
Bihar	8	2	5
West Bengal	-	-	1
Sikkim	1	-	-
Orissa	4	-	2
Andhra Pradesh	-	4	-
Assam	2	-	-
Nagaland	1	-	-
Mizoram	1	-	-
Meghalaya	2	-	-

Results

Distribution of RJF Gallus gallus in India

The current distribution of RJF based on the rule model is *c*652,000 km^2 (FIG. 1). The species is present in 205 Districts in 21 range states in India. Of the 255 PAs that occur within the RJF distribution range in India, 190 PAs (31 NPs and 159 WSs) have reported presence in their areas. During March 2007, there were 209 individuals (74 males: 80 females: 55 chicks) RJF in captivity in the various zoos and pheasantries in the states of Andhra Pradesh, Delhi, Haryana, Himachal Pradesh and Uttar Pradesh.

As there are no population estimates for RJF in the Pas, an encounter rate method was used. A total of 38 trails covering 358 km were traversed taking 546 man hours. The encounters rate was pooled together for similar habitats (TABLE 2). The encounter rate was the highest for the summer months in the Shivalik Region. This area also had the highest variance around the estimate, suggesting that more sampling is needed for the area.

Morphological traits

In total, 563 RJF (293 males and 270 females) were sighted during the field surveys. All males had the presence of yellowish hackles, slatey black thin tarsus and white ear lobes in 7% (n= 5) of the central region, while other areas had pinkish ear lobes. The tail carriage was difficult to characterise, but the presence of the sickle feather was prominent in all cases. In 30% of females, the comb was either rudimentary or absent, while in the rest, classification was not possible. This was mainly due to cryptic colouration and behaviour. Eclipse plumage was also observed in RJFs (n = 70), however captive populations that were examined at the Morni Hill Pheasantry during the period July – September and these also had eclipse plumage.

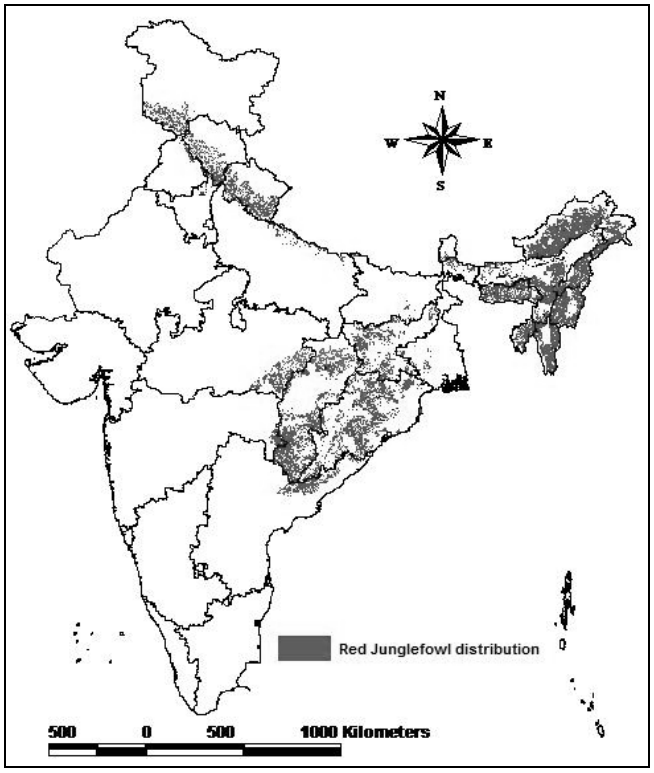

FIG. 1 Current distribution of Red Junglefowl in India.

Isolation of genomic DNA from different tissues

A total of 92 samples of RJF and 25 samples of domestic chicken were collected from various parts of India (TABLE 1). Of these 50 samples were from wild and 42 samples from captivity. 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).

TABLE 2 Encounter rate (No/km) for Red Junglefowl in various habitat types in India.

Habitat types	Area	Months	Transects (no, km)	Birds (mean no per km ± s.e)
Mangroves	Bhittarkanika	Nov	4, 9.8	1.36 ± 0.29
Moist mixed forest	parts of Orissa & Udanti WS	Nov-Dec	10, 46	0.17 ± 0.09
Dry deciduous	parts of Andhra Pradesh	Dec-Jan	16, 103	0
Moist mixed forest	Meghalaya & Assam	Feb-Mar	18, 72	0.47 ± 0.26
Grassland & Woodland	Assam floodplains	Mar-Apr	18, 40	1.43 ± 0.21
Shivaliks	Uttarakhand	May	12,19.2	5.06 ± 1.26
Himalayan foothills	Himachal Pradesh	Jun-July	8, 18	0.69 ± 0.30

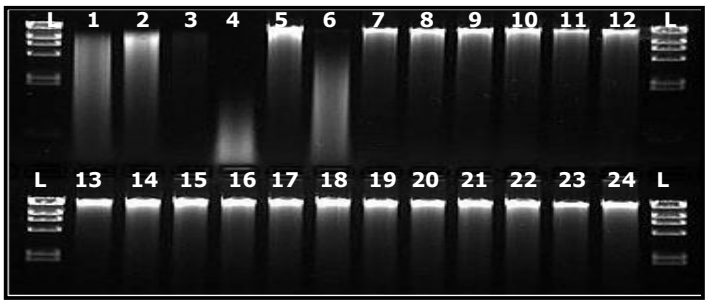

FIG. 2 Gel electrophoresis (0.8% agarose) of extracted DNA, L - 1Kb ladder, Lane 1 to 12 RJF samples, Lane 13 to 24 domestic chickens.

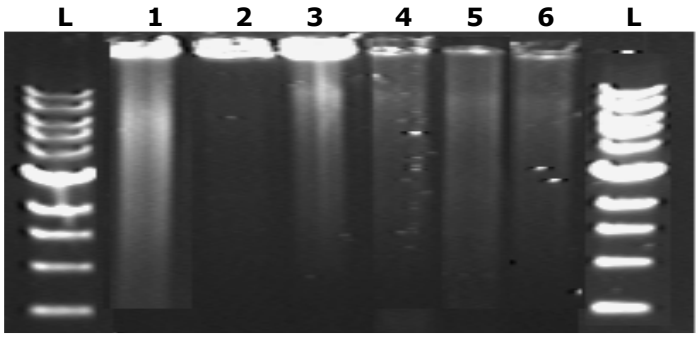

FIG 3 Gel electrophoresis (0.8% agarose) of extracted DNA, L - 1Kb ladder, lanes 1 = blood, 2 & 3 = tissue, 4 = pulled feathers, 5 = fallen feathers and 6 = egg shell.

Optimisation of PCR assay for microsatellite genotyping

PCR cycling condition was optimised for all microsatellite loci and the optimised annealing temperature are given in TABLE 3. Annealing temperature for each locus was optimised in a gradient PCR (FIG. 4) and seven microsatellite loci were amplified in extracted DNA samples. The amplification success rate showed a discrepancy with sample types as the blood and tissue sample showed a higher success rate, followed by feathers while with egg shell, the success rate was least (FIG. 5). The average allele sizes for microsatellite loci was similar to those reported by Weigend (*pers. comm*.).

TABLE 3 Microsatellite markers used.

Marker	Chromosome	Allele Range	T_A
ADL0268	1	102-116	60^0C
MCW0206	2	221-249	60^0 C
LEI0166	3	354-370	60^0 C
MCW0020	1	179-185	60^0 C
MCW0037	3	154-160	64^0 C
LEI0192	6	244-370	60^0 C
ADL0112	10	120-134	58^0 C
MCW0295	4	88-106	60^0 C
MCW0067	8	176-186	60^0 C
MCW0104	13	190-234	60^0 C
MCW0111	1	96-120	60^0 C
MCW0034	2	212-246	60^0 C
MCW0222	3	220-226	60^0 C
LEI0094	4	247-287	60^0 C
MCW0216	13	139-149	60^0 C
MCW0081	5	112-135	60^0 C
MCW0330	17	256-300	60^0 C
LEI0234	2	216-364	60^0 C
MCW0103	3	266-270	64^0 C
MCW0098	4	261-265	60^0 C
MCW0284	4	235-243	60^0 C
MCW0069	E60C04W23	158-176	60^0 C
MCW0016	3	162-206	60^0 C
MCW0078	5	135-147	60^0 C
MCW0014	6	164-182	58^0 C
MCW0183	7	296-326	58^0 C
MCW0123	14	76-100	60^0 C
MCW0165	23	114-118	60^0 C
MCW0248	W29	205-225	60^0 C
ADL0278	8	114-126	60^0 C

Discussion

The present state-wise distribution obtained by 'presence' only data is the same in comparison to the historical distribution of Hume & Marshall (1879), Ali & Ripley (1983) and Madge & 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 the Pench River (R. Jayapal *pers. comm*). Ali & Ripley (1983) demarcate the southern most distribution of RJF to be near Rajahmundry but there are records by Nagula et al. (1997) of RJF in Eturnagaram WS. The present survey did not record presence of RJF within these sanctuaries, but the species was recorded on the west bank of the Godavari River at Pollavaram and hence it is proposed that further effort is required in order to confirm its status as it forms the southern most distribution for this species. From the rule based model, it is clear that the distribution is split into two broad regions the north – northeast region and central region (FIG. 1). The distribution from the north to the northeast region is connected through Nepal, while the central region is disconnected from the northeast as there is no forest connectivity in Bangladesh from its eastern side to its mangrove forest. To better understand the RJF habitat relationship, habitat suitability modelling will be taken up with the help of Landsat imagery using geographical and habitat variables. From this predictive distribution the analysed genetic data will be overlaid so as to give different regions with respect to pure and hybrids.

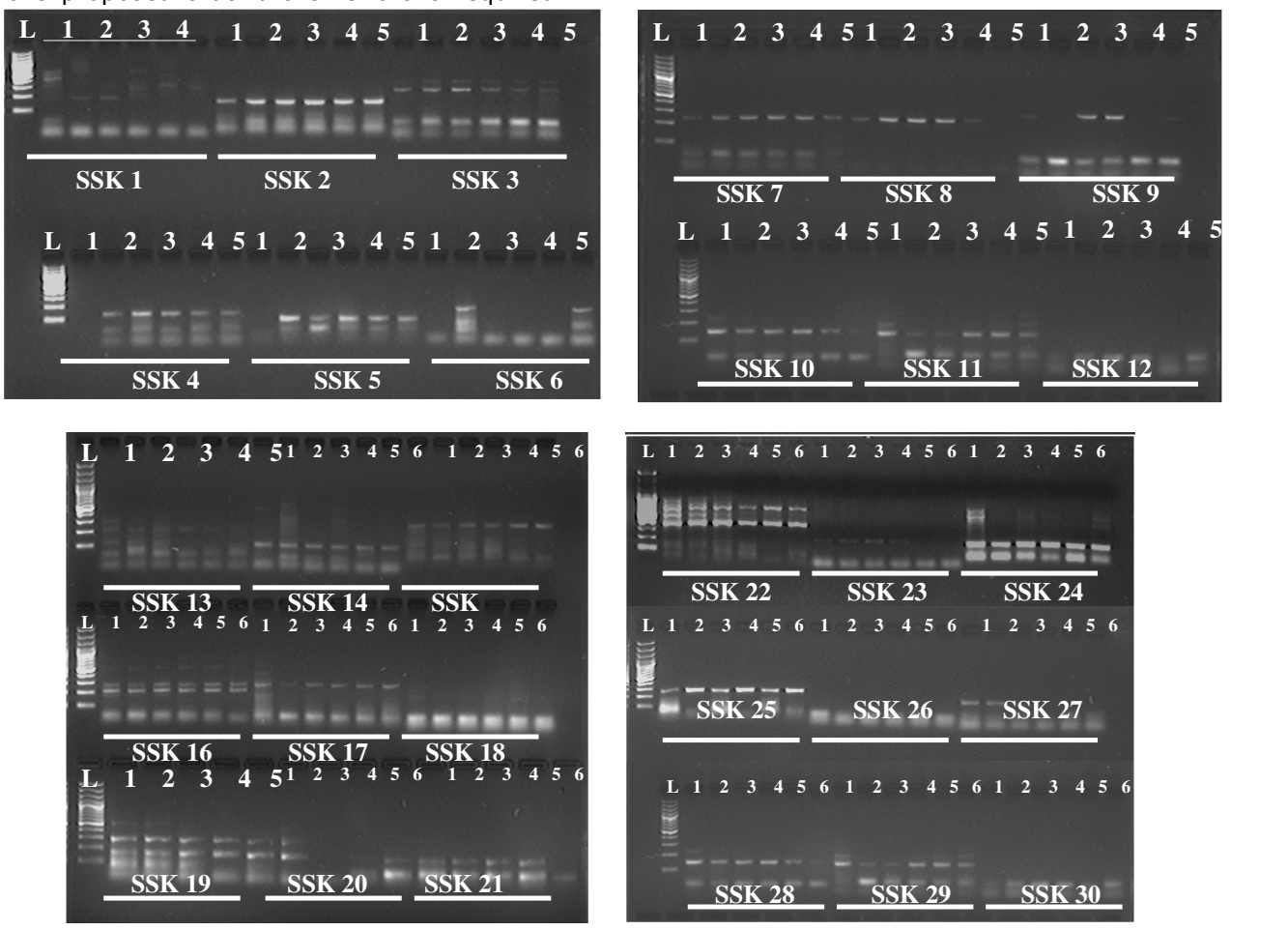

FIG. 4 Standardisation of annealing temperature for amplification of thirty microsatellite loci, L - 100bp, annealing temperature 1 - 55^{0}C, 2 - 56.7^{0}C, 3 - 59.3^{0}C, 4 - 62.4^{0}C, 5 - 64.3^{0}C and 6 - 65.0^{0}C.

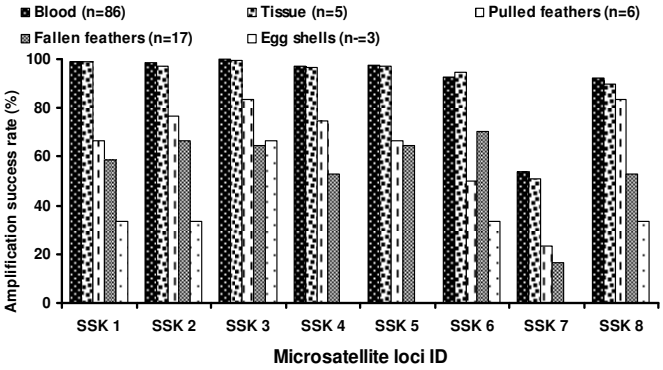

FIG. 5 Amplification success rate in reference of sample types

Due to the varied habitat in the distribution range there were constraints in transect 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, which will give an abundance estimate.

In this study different samples were used for DNA extraction. The blood and tissue proved to be very good samples types for DNA extraction. 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 & Appa Rao, 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. 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 Weigend (*pers. comm*.). 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 need 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 seven microsatellite loci, these microsatellite markers, as well as another 23 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 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's comb, leg colour, horizontal body posture and tail carriage, simpler and a shorter call, it may be necessary to correlate the genetic purity and the physical traits in order to identify pure RJF.

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.

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