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Comparison of different parameters of invasive and non invasive sampling methods for microsatellite genotyping: a case study from Red Junglefowl and its application for other Galliformes

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ABSTRACT:

In studies dealing with genetic and disease prognosis, biological sampling is a prerequisite. Earlier, blood and muscle samples were taken after live captures. These invasive procedures are of considerable risk when sampling endangered species. Therefore, non invasive samples like shed feathers/hairs, faeces and hatched or predated egg shells are the alternative source for genetic study. We collected 18 Red Junglefowl feather samples (plucked feathers n= 6; shed feathers n=12) across its northwestern distribution range in India. The DNA yield was compared with other sample types *i.e*. blood, egg shell and faeces (n=6 for each sample type). We found a gradient in DNA yield as blood (770 µg/ml) > plucked feathers > shed feathers > egg shell = faeces (30 µg/ml). DNA extracts from feathers were amplified for four microsatellite loci. Three samples (17%) for all 4 microsatellite loci, four samples (22%) for 3 and 2 loci each, and seven samples (39%) for 1 locus were successfully genotyped. We found that the successful amplification of multilocus genotypes were dependant on the condition of the starting material and the type of sample. There are few studies that have compared the quality and quantity of DNA being produced through varying degree of invasiveness. None have considered effort (time) and the cost involved in procuring the samples from field and processing samples in lab. We scaled all the aspects of cost and efforts from 1 (low) to 10 (high) and here presenting a comparative analysis for the efficacy of invasive and non-invasive sampling methods.

Keywords:
Invasive and non-invasive sampling, feathers, genotyping and Red Jungle fowl.

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INTRODUCTION

Red Junglefowl (RJF) [*Gallus gallus*] is a common pheasant species widely distributed in a variety of habitats within India (Ali and Ripley, 1983). They are ground dwelling and nesting birds preferring a mosaic of habitats with good undercover, where they prefer to breed during the summer months from March to June (Johnsgard, 1986).The RJF is widely distributed and its five subspecies are spread from the Indian subcontinent eastwards across Myanmar, South China, Indonesia to Java. In India, two sub-species occur, the type specimen are *Gallus gallus murghii* and *Gallus gallus spadiceus* (Ali and Ripley, 1983; Fernandes *et al.*, 2008 and 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 north eastern parts of India. 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 area. There were concerns regarding issues of hybridisation of wild RJF with domestic chicken, leading to genetic endangerment of the species (Brisbin, 1995). In order to investigate these concerns, we have been collecting different sample types through various methods and extracting genomic DNA (g DNA). Less invasive methods such as plucked feather (Taberlet and Bouvet, 1991 and Fiona *et al.*, 2008) and non invasive methods which use faecal droppings and eggshells have increased over time (Segelbacher *et al.*, 2001 and Bush *et al.*, 2005). Though, there is constant limitation with the DNA yield with these sample types but are importance as being a vital source of genomic DNA. Earlier, Bush *et al.*, (2005) has investigated the quality and quantity of DNA being extracted through various degree of invasiveness in temperate region but there are no studies within the tropical system. Therefore, we had collected different sample types using various methods of invasive and non-invasive sampling techniques and compared them for some essential parameters *viz.* effort and cost involved in getting samples from field and processing them in lab and yield of DNA from each sample type.

MATERIALS AND METHODS

Sample collection and DNA extraction

We sampled six sites within the northwestern distribution range of RJF from February to May 2008 in the States of Jammu and Kashmir (n= 2), Himachal Pradesh (n= 1), Haryana (n= 1) and Uttarakhand (n= 2). Live trapping was attempted using leg-hold noose (n= 10) (Bub, 1991 and Ramesh *et al.*, 2008) in combination with fall net (n= 2). From the live trapped bird, blood was drawn from brachial vein (n= 6) and stored in DNA zol BD (Mackey *et al.,* 1996). A single feather was plucked (n= 6) and preserved in 70% ethanol (www.gallus.forestry.uga.edu). Shed feathers (n= 12) of RJF that were collected during field surveys were stored in plastic zip lock bags. Incidental collections of hatched eggshells (n= 2) and faecal droppings (n= 3) were made. A tissue sample that was collected from a predated RJF specimen outside the study area was used as a comparative reference sample during the analysis. The g DNA was extracted from individual feather using Qiagen DNeasy tissue kit (Qiagen, Germany) following manufacturer's protocol with the following alterations: (i) addition of 100 mg/ml DTT solution in the lysis buffer, (ii) digestion was performed overnight at 55^0C in a shaking water bath and addition of ice chilled ethanol for better precipitation, and (iii) DNA was finally recovered in 30-40 µl of elution buffer and stored at -20^0 C. DNA zol BD based extraction protocol was used to extract DNA from blood while for tissue and eggshells the Qiagen DNeasy tissue kit (Qiagen, Germany) was used following manufacturer's protocol. The DNA isolated from all the above sources were quantified using UV-Visible spectrophotometer (GeneQuant *Pro*, Amersham Biosciences) and compared across different sources.

Microsatellite Genotyping

Individual samples extracted from feathers were genotyped twice for increasing the efficiency of the results with four (CA) n dinucleotide microsatellite loci *viz.,* MCW-0295, MCW-034, LEI-111 and LEI 192 (Sharma., 2006). PCR was carried out in a 10 µl reaction volume in an Applied biosystem thermal cycler (2700 and 2720) and the reaction mixture consisted 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 80ng of gDNA. PCR profiles consisted of 2 min initial denaturation at 94^0C, followed by 35 cycles of denaturation at 94^0C for 45 sec, annealing at specific temperature (MCW 295- 55^0.0C, MCW-111-64.9^0C, LEI-192- 62^0.0C, MCW-034- 64.9^0C) for 45 sec and extension at 72^0 C for 2 min with a final extension for 10 min at 72^0 C. PCR products were resolved on 2% agarose gel containing

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ethidium bromide (4µl/100ml). To avoid cross contamination during pre and post PCRs, all pipetting were carried out using aerosol resistant filter pipette tips in separate rooms.

Parameters set for Analysis

For analysis, we compared across sampling methods with time (effort to obtain and to process a sample) and costs involved in field and lab. All the aspects of cost and effort were scaled from 1 (low) to 10 (high). For the effort in the field, the minimum and maximum time taken for a successful sample, *i.e.,* live-trapped (blood/ plucked feather), shed feather, faecal droppings and collection of hatched egg shells while operational costs that include personnel and field expenditure were summed and then scaled. This also includes collection of faeces when the trapped bird defecated and opportunistic collection of hatched egg shells. Travel costs to different field sites were not included in the analysis. We averaged effort and costs across sites for collection of field samples. Similarly laboratory efforts included processing of a sample for microsatellite genotyping (DNA extraction, gel electrophoresis, PCR amplification and genotyping) and the cost included personnel and the approximate expenditure for processing one sample for genotyping but not including infrastructure and equipment costs of the lab.

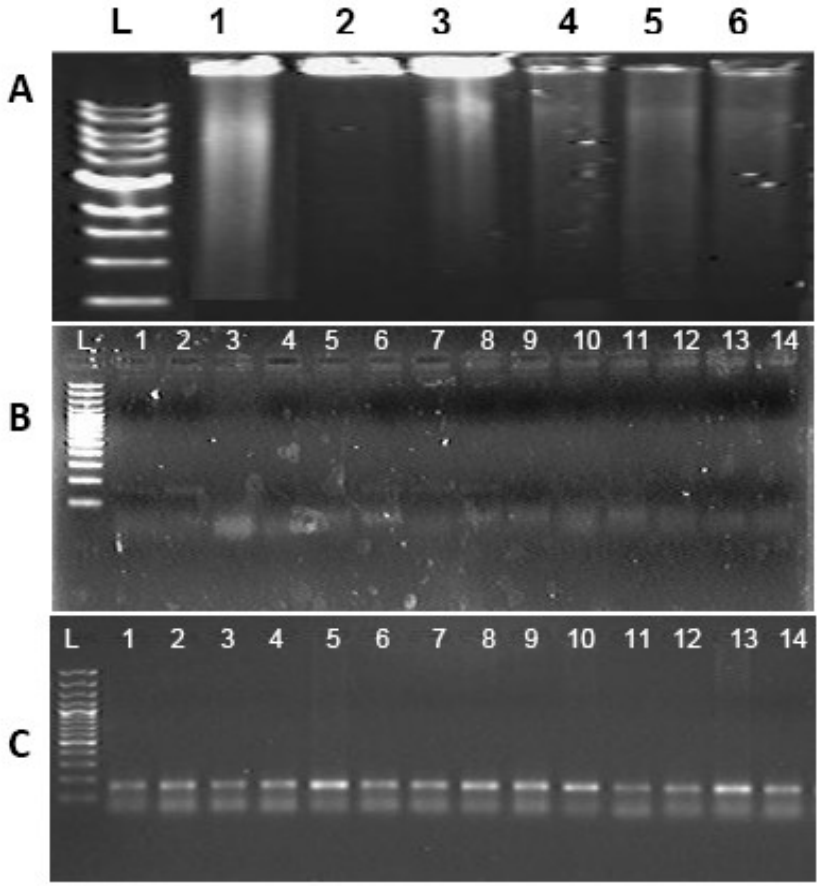

Figure 1 Gel Electrophoresis of extracted DNA and amplified PCR products

A - Gel Electrophoresis (0.8% agarose) of extracted DNA (L- 1Kb DNA ladder, Lane- 1 DNA from blood sample, Lane- 2, 3 DNA from tissue, Lane - 4 DNA from plucked feather, Lane-5 DNA from shed feather, Lane-6 DNA from egg shell membrane)

B - Gel Electrophoresis (2% agarose) of amplified PCR products from Feathers samples (I PCR) (L- 100 bp DNA ladder, Lane 1-10 Plucked feathers, Lane 11-14 Shed feathers)

C - Gel Electrophoresis (2% agarose) of amplified PCR products from Feathers samples (II PCR) (L- 100 bp DNA ladder, Lane 1-10 Plucked feathers, Lane 11-14 Shed feathers).

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Table 1 A comparison of genetic sampling methods with some essential parameters

Technique	Type of Sample	Field effort (min) [1]	Field cost (₹) [2]	Lab effort (min) [3]	Lab cost (₹) [4]	Average Score (1 to 4) (%)	DNA concentration (µg/ml) # [5]	Efficacy Rank
Invasive (Live capture)	Blood	1120 (5)	1633 (5)	3315 (1)	723 (1)	32.5	770	1
	Plucked Feathers	1120 (5)	1633 (5)	1035 (7)	763 (3)	52.5	67	2
Non-Invasive	Shed Feathers	240 (1)	700 (1)	1275 (9)	891 (7)	45	34	3
	Eggshell *	Nil	Nil	1515 (10)	1001 (10)	Nil	30	-
	Faeces*	Nil	Nil	1515 (10)	1001 (10)	Nil	30	-

Figures in parenthesis denote scores (1 = low and 10 = high), Denoted effort and costs are calculated for one sample.

*** Field effort and cost not considered as the collection of these samples types were incidental during the study and such samples are usually very difficult to obtain.**

 # the values presented in the Table is the mean of six samples of each sample type.

RESULTS AND DISCUSSION

All the sample types were extracted successfully for g DNA but there was a gradient in the DNA yield (mg/ml) (**Table 1**) *i.e.* blood (770 µg/ml) > plucked feathers (67 µg/ml) > shed feathers (34 µg/ml) > egg shell (30 µg/ml) = faeces (30 µg/ml). DNA concentration (µg/ml) values presented in table are the mean of six samples of each sample types to reduce the error during quantification through spectrophotometer. The extracted DNA was visualized on gel for quality assessment (**Figure 1- A**). For genotyping with four microsatellite loci, we followed the multiple tube approach and a second or third PCR for shed feathers in order to obtain visible gel bands that could be scored (Gagnex et al., 1997; Kohn et al., 1999 and Morin *et al.,* 2001) (**Figure 1 B & C**). In genotyping all DNA extracts of shed (n=12) and plucked (n=6) feathers, three samples (17%) for all 4 microsatellite loci, four samples (22%) for 3 and 2 loci each, and seven samples (39%) for 1 loci were successfully genotyped (**Table 2**). The remaining samples (n=11) yielded either no PCR product (n=6) or showed weak gel bands (n=5) and therefore were excluded from further analysis. Comparison of invasive and non invasive sampling methods with various sample types has been shown in Table 1 considering time and effort as parameters for field and lab.

Although all sample types yielded gDNA, there was a large gradation in the concentrations of DNA from invasive and non invasive methods, which are in accordance to many studies (Taberlet and Bouvet, 1991; Fiona *et al.,* 2008; Segelbacher and Steinbrück *et al.,*2001 and Bush *et al*., 2005) (Table 1 Column 5). In terms of quality and quantity of g DNA obtained, blood was considered to be better when compared to feather follicle and egg membrane (Fig 1A and Table 1). The lower yield of gDNA from shed feather, faeces and eggshell could be due to enhanced microbial activity causing decay since sampling was undertaken during post winter and summer seasons. We found that samples with low DNA quality have

Table 2 Amplification success of Microsatellite loci from Feathers (Shed *vs.* Plucked feathers)

No. of loci amplified	No. of samples (%)	Type of Feather Samples	
		Shed feathers	Plucked feathers
1	7 (39)	4	3
2	4 (22)	3	1
3	4 (22)	3	1
4	3 (17)	2	1
Total	18	12	6

a higher probability of amplification failure and need a multiple tube approach to get scorable bands on gel. Various methods address these problems, including the 'multitubes approach' (Navidi *et al*. 1992 and Taberlet *et al*. 1996). In fact Jason *et al.* (2005) stated the locus size effects on amplification success, allelic dropout and error rates in non-invasive genotyping studies while considerable amplification success was also achieved by few researchers using non-invasive samples (Kohn *et al*., 1995; Gerloff *et al*., 1995 and Vidya and Sukumar, 2005). Comparison of different sampling methods in the field suggests that samples obtained through non-invasive methods were much efficient than invasive methods (Table 1, Columns 1 & 2). Though the field efforts were primarily focussed on gathering information on the ecology of the species through observations, incidental finds of faecal droppings, eggshells and a predated RJF were collected to supplement information and subsequently used for extracting DNA. As sampling was undertaken during the breeding season, it could have facilitated the availability of shed feathers and egg shells. The field identification of the shed feathers for RJF was possible because of its distinguishable features (Morejohn, 1968). However, for field identification of shed feathers for other galliformes species, advanced techniques (Sivakumar *et al.,* 2007) would be required for confirmation. Similarly, hatched eggshells were collected from where breeding observations were made. Though the cost and effort in field were higher in case of invasive methods, it yielded better results with low cost and effort in the lab. Whereas in case of non invasive methods, the cost and effort were lower in field and higher in lab for shed feather samples due to consequent increase in the cost and effort for processing samples to obtaining g DNA.

CONCLUSION

Based on this study, we may conclude that there is a certain trade-off in deciding which sampling method is the most appropriate for galliformes. Invasive sampling method is suitable for the common or abundant species. The efforts in field may increase manifold for species that are shy, elusive, inhabit dense habitats, and have limited distributional range or low in abundance. Hence, in order to sample these species, efforts could be made to obtain shed feathers though cost and effort increases in the lab and quantity of DNA may be low.

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