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RFLP assessment for genetic diversity among Indian Poultry birds of Himalayan Region

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Abstract
As a result of many years of domestication and breeding, a wide variety of chicken breeds exist today. However, because of many malpractices an increasing number of local breeds are under threat of extinction rendering many valuable genotypes and traits at risk of being lost. The genetic erosion of these local breeds may lead to the loss of valuable genetic variability in specific characteristics that are momentarily unimportant in commercial breeding strategies. The present work deals with applying the RFLP Technique on a selected gene (IGF1) to look for the genetic diversity within 10 individuals of Red Fowl and Black Fowl of Himalayan Region. The work is further extended on bioinformatics level wherein the sequence obtained for one of the randomly selected amplicon was locally aligned to search its similarity with the closest sequence(s) for tracing the phylogeny.

Keywords: RFLP, Genetic diversity, Red fowl, Black fowl, Himalayan region, Phylogeny

Introduction
Domestic chickens are believed to descent from multiple domestication events over the last 8000 years, mostly from Red Jungle fowl (Gallus gallus) in South-East Asia and, to a lesser extent, from Gallus sonneratii in south-west India and Gallus lafayetii in Sri Lanka. Later, as human migrated the chicken also spread to Europe and Africa along trade routes. (C. M. Lyimo *et.al* 2014) [4].
Red jungle fowl are the wild ancestors of all domestic poultry, although the rooster is said to be more brilliantly colored than its tame relative. The vibrant male has long, golden-orange to deep-red crown and neck feathers, and a dark metallic-green tail with a white tuft at the base. The underparts are a dull black while the upperparts are a combination of glossy blue-green, rich dark red, maroon-red, fiery orange, rufous and blackish brown. The colorful cock also has vivid scarlet-red facial skin, throat, two lappets and heavily dented fleshy crest, and red or white ear patches on the sides of the head.
The male of Black Fowl has a black cape with ochre spots and the body plumage on a grey ground colour is finely patterned. The elongated neck feathers are dark and end in a small, hard, yellowish plate; this peculiar structure making them popular for making high-grade artificial flies. The male has red wattles and combs but not as strongly developed as in the red jungle fowl. Legs of males are red and have spurs while the yellow legs of females usually lack spurs. The central tail feathers are long and sickle shaped. Males have an eclipse plumage in which they moult their colourful neck feathers in summer during or after the breeding season. (Morejohn, 1968) [7].
Molecular genetic characterization explores and studies the polymorphism in selected protein molecules and deoxyri-bonucleic acid markers in order to measure genetic variation at specific points in the population. In poultry, different genetic marker systems or techniques have successfully been used for estimation of genetic variability. They were DNA fingerprints, RAPDs, and microsatellites (Rudresh *et.al* 2015).
Determining genetic diversity can be based on morphological, biochemical, and molecular types of information (Goncalves *et al*., 2009) [5]. However, molecular markers have advantages over other kinds, where they show genetic differences on a more detailed level without interferences from environmental factors, and where they involve techniques that provide fast results detailing genetic diversity (Binneck *et al*., 2002) [2].
The status of poultry rearing in Himalayan region of India is not encouraging, with the current total population of poultry birds limited to just a few thousand. There are many constraints contributing to this, including limited availability of feed, lack of subsidies, religious sentiments, lack of availability of suitable germplasm, limited poultry feed ingredients, and poor knowledge regarding poultry farming. Furthermore, the housing of poultry is difficult in this cold and arid climate (Biswas *et al*., 2010) [1].
PCR restriction fragment length polymorphism (RFLP) assay is a two-step reaction to identify multiple species after restriction enzyme digestion of PCR amplified DNA sequence (Haider *et al*., 2012) [6].

Materials and Methods
Sample collection
2mL blood was collected in K3 EDTA tubes from 10 individuals of Red and Black Fowls of Himalayan region each from fast white broiler and Rainbow rooster multicolored dual purpose birds. The supplier of the samples was Indbro Research & Breeding Farms Pvt Ltd. All the samples were stored at –20 °C.

Genomic DNA Isolation, purification and qualitative estimation
Genomic DNA was extracted by the Bunce's method in which the anti-coagulated blood was treated with a solution of Tris HCl, Sucrose and $MgCl_2$. Further EDTA and NaCl solution was added to the pellet followed by the subsequent treatment with Sodium acetate and ice cold chloroform. To the supernatant obtained an equal volume of ice cold iso propanol was added for proper DNA precipitation. For purification the precipitated DNA was incubated at -20^{o}C overnight and centrifuged at 6000rpm/10min.The pellet was washed with 1

ml of 70% ethanol and re-centrifuged. The air dried DNA pellet was dissolved in100µl of 1xTE buffer.
The agarose gel electrophoresis was carried out to check the integrity and quality of the DNA yield. All the samples were run on 0.8% Agarose at 100V for 45 min. Furthermore the spectrophotometric readings were taken to obtain the ratio at wavelengths of 260 and 280nm. All the samples were then diluted to 25 ng/µl each for using them in PCR.

Primer designing and amplification
Specific primer was designed for the IGF1 gene target region using Primer BLAST and the target region was amplified. The mixture of the PCR reaction had a final volume of 25 µl and contained 50 ng of genomic DNA (2µl), 5 µl of Taq buffer, 1 µl of 1.5mM $MgCl_2$, 2 µl of 0.2 mM/ µl dNTP, 2 µl of 20 pmol/ µl of each primer, 2 µl of 1.00 Unit/ µl *Taq DNA polymerase* ("Fermentas") and 9 µl of nuclease free water. The amplification was performed in 'Bio-Rad Thermo cycler' using the following cycling parameters: pre denaturation (94°C) 5 min, 30 cycles of denaturation (95°C) 45 sec, annealing (55°C) 30 sec, extension (72°C) 1min, Final extension (72°C) 7 min.

Restriction Digestion
The amplified product was subjected to restriction digestion activity of enzyme Hinf I whose recognition sequence is GANTC. To 7 µl of each of the 20 amplicons 2 µl of Hinf I and 2.5 µl of 10X Assay Buffer were added and the volume was made upto 25 µl by adding distilled water. The enzymatic activity was allowed to run for 1 hour with proper incubation at 37ºC.The enzymatic activity was stopped by adding 5 µl of 6X Loading dye. The digests were run on 1.8% agarose stained with Ethidium Bromide along with 100bp ladder.

Sequencing and Sequence Analysis
Out of the many amplicons of the digested fragments obtained in gel doc, one from the 9th sample was randomly selected for sequencing by Sanger's method using ABI Prism 377 DNA sequences. The sequence obtained was further analyzed by BLAST to look for the sequence most identical to it.

Results and Discussion
In the PCR-RFLP, species detection is done by PCR amplification of targeted gene, sequence analysis and subsequently digested with restriction enzymes which cleaves the DNA fragments at specific site results in characteristic band patterns depending on the particular enzymes used. The resulted characteristics band pattern can be used for identification or differentiation of species. PCR reaction and subsequent digestion with restriction enzymes has been found to be versatile tool for identification of more than one species.
In this study, self-designed universal primer pair of IGF1 gene region was employed for successful amplification of 527 bp desired DNA fragments from DNA extracted from 10 chicken samples of Himalayan region. The highly conserved nature of IGF1 gene DNA sequence in different species of animals found to be suitable for design of universal primer pairs for PCR amplification.
The primer designed and used for amplification of target region of IGF1 was as follows:

Table 1

	Sequence (5'->3')	Template strand	Length	Tm	GC%
Forward primer	ATAGAGCCTGCGCAATGGAA	Plus	20	59.82	50.00
Reverse primer	ACATACAGCCATTTTCCAGATCAC	Minus	24	59.36	41.67

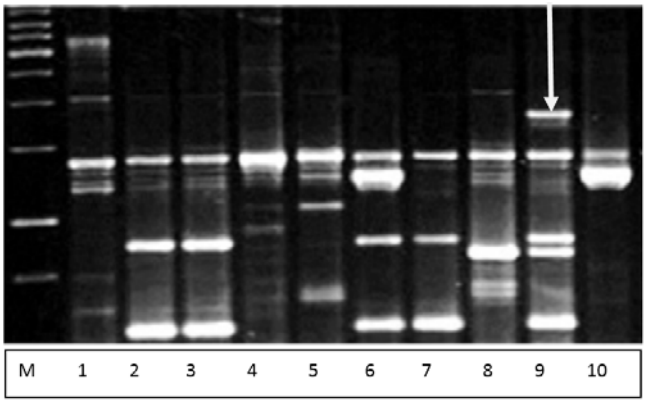
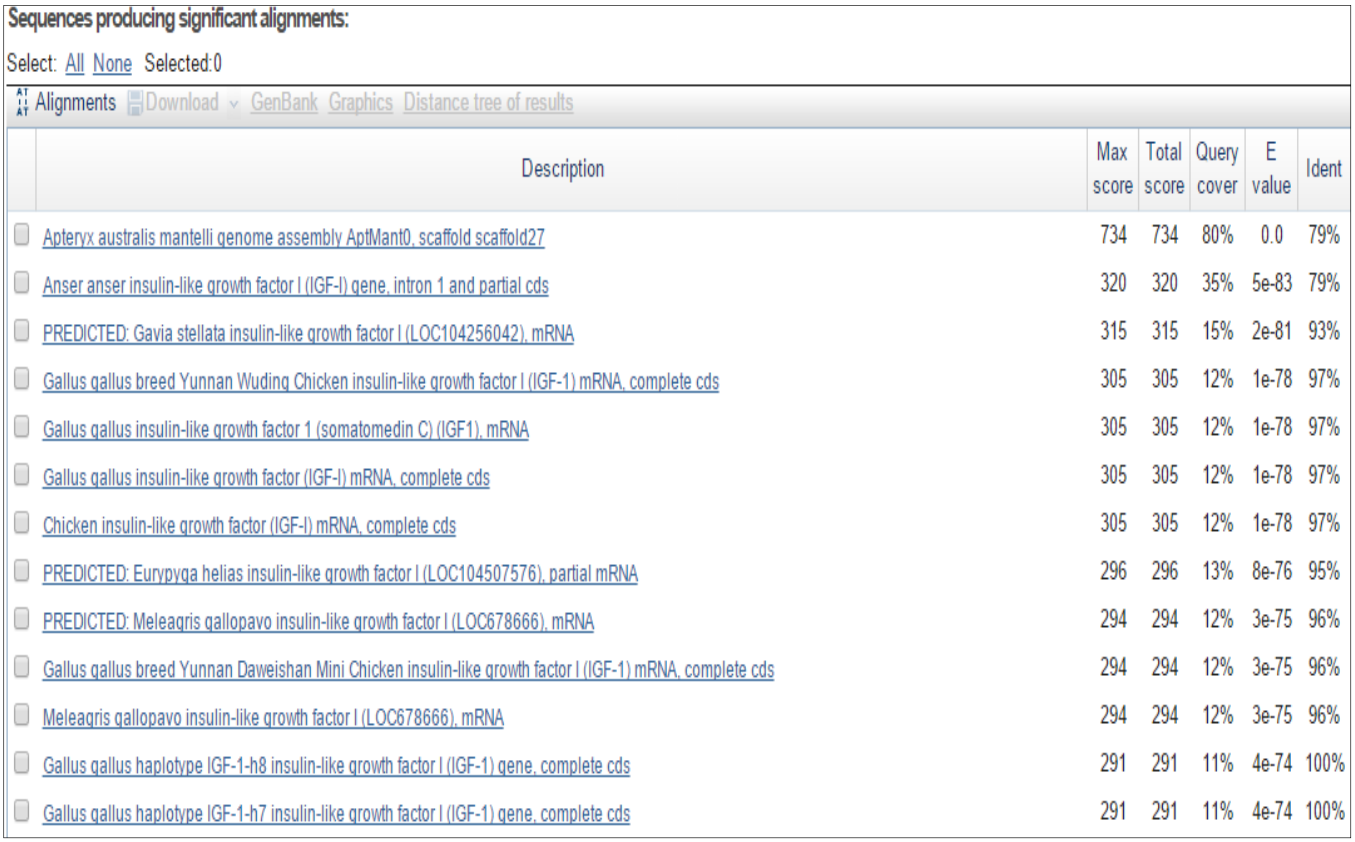


Fig 1: Band pattern of IGF1 gene digests of Hinf I in Red and Black Fowls of Himalayan Region (arrow indicate the selected amplicon for sequencing)

The variability observed in IGF1 gene has been used to identify the species The RFLP results signify that sample number 2, 3 and 7 have almost identical band pattern indicating them of belonging to same genera and species. The band pattern of rest all others are different from one another.
This data compiled to show genetic variation among different varieties of chicken is quite useful and can be used by breeders to sort out the problem of variation in body weight of chicken and hence select desired species of chicken for breeding. The selection and optimization of molecular markers is a fundamental step towards full success in genetic studies.
Poultry forms are one of the major sources of providing chicken meat in high populated country like India. Growing big size chicken will be one of the great steps in order to increase food productivity. In this study genetic relationships information generated through RFLP by selecting the primers with high discriminatory power would be useful for utilization for future breeding purpose.

Sequence Analysis of selected amplicon by BLAST
AGT CAC TGA ATG TAG TCC CCT ATT ATA GAA GGT CCC ATA CAA ACT CTT CAG TAA ATT CAG CAA GTT CTA TCT TAA AAC ACT TTA TTT TGT TTT AAT GTT TTC ATT CTT AAA GTG TCT GGA CAA CCA CAG AGT CTT AAT AAT TTA TTT TTT TCC TCC TGG GCA AAA TTT CCT TTC ATC TAG TTA ACA GCC ATT TAT TCT TTT GAC AGC GCT GTT GGT TTT GTT TGT TTG TTT GTT TGT TTG TTT GTT TGT TTT TAA GTT TAA ATA GCT CTT ATA TAC CAT TTC CAC ATA ACC CAG TGC ATT GAC AGA CAA CTG TCA TTT TCC CTA TTA GCC AAT CTG GGC AGA AAC AAA TCA ACA TAA GGT TGT CCA GTC TTC CTT ACC CTA ACT ATT CTG GAC TTC CTG GGT TTC TTC ATC TTT CTA CTG CAC CAT

TCC ATA TCC TCC TCT AAC AAG AGC AAC TAT AAA TCA TAA TTC CTG CAA GGT TTT CTA ACT ACA TGT GAA TGT GAA CCA AGA ATA CTT GCT AGA TTA AGT CAT CAG TTT ACC AAA TGT TAA GTC TAG AAT ATG TAG ATT GCA GTC TAT CCT GAT ACT GCA TAT ATT GTC TAA CAA ATT ACA GTA CAT ATG CAC TAT CAG TTT ATG CAG GTG CAG GAT TCT TTT TTT TTT AAG ACT CTG TTG GGA AAC TGA CAC AGT ATT TTT GCT TCC TTG GCA GGT GAA GAT GCA CAC TGT GTC CTA CAT TCA TTT CTT CTA CCT TGG CCT GTG TTT GCT TAC CTT AAC CAG TTC TGC TGC TGC CGG CCC AGA AAC ACT GTG TGG TGC TGA GCT GGT TGA TGC TCT TCA GTT CGT ATG TGG AGA CAG AGG CTT CTA CTT CAG TAA GTC AAC CCT TTC ATT CAA CTG CTA AGT TTT TCC ATG TCA TCC ATT AGA GTT TGT AAC TGC ATT AAG CAA GCA TAG GAA TCA TCC AAC GAG GCT GCT AGG TAC CAC CAA GTA TCC TCG ATC TCA GAG ATG CCC AAG ACT TCA ATG ACC ATT AAA TCT CCG TTA AGA ATA GAT ATG TTC CTA TAT TCA GTT CTC TGA GAT TTT GAT GAA TTT TAC TTT TCA GGC ATA CTA TTG TTT TTA ACT ACT CAC CAA CTG CTT CTA TCA CAC CTG CTA TGT TAT TGC TTC TCC TGA CCT ACC ACT GAA AAT AAG GGA ATG AAA GGA CAC ACT AGA CCC AAG ACA GGG CAG AAC TGC ACG TAC CTA TTA AGT CAG ACT ATT CGT AAA TAG TTT AAA TAC ACA TTG TAT CAT ATT AAA AAC ACA CAA ATC TTC AAC ATC TGG TTT GAA AAA AAG GAA AAG CTG GAA GGC AGC AAG CAG AAA GGC ACT GCA TCC AAG TAT CCA CAT ACT ACA TCA GTT AAG TAC AGG TGA AGG AAA ATG CTT TCA TGG TGA GAT AAA AAA G



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident
Apteryx australis mantelli genome assembly AptMant0, scaffold scaffold27	734	734	80%	0.0	79%
Anser anser insulin-like growth factor I (IGF-I) gene, intron 1 and partial cds	320	320	35%	5e-83	79%
PREDICTED: Gavia stellata insulin-like growth factor I (LOC104256042), mRNA	315	315	15%	2e-81	93%
Gallus gallus breed Yunnan Wuding Chicken insulin-like growth factor I (IGF-1) mRNA, complete cds	305	305	12%	1e-78	97%
Gallus gallus insulin-like growth factor 1 (somatomedin C) (IGF1), mRNA	305	305	12%	1e-78	97%
Gallus gallus insulin-like growth factor (IGF-I) mRNA, complete cds	305	305	12%	1e-78	97%
Chicken insulin-like growth factor (IGF-I) mRNA, complete cds	305	305	12%	1e-78	97%
PREDICTED: Eurypyga helias insulin-like growth factor I (LOC104507576), partial mRNA	296	296	13%	8e-76	95%
PREDICTED: Meleagris gallopavo insulin-like growth factor I (LOC678666), mRNA	294	294	12%	3e-75	96%
Gallus gallus breed Yunnan Daweishan Mini Chicken insulin-like growth factor I (IGF-1) mRNA, complete cds	294	294	12%	3e-75	96%
Meleagris gallopavo insulin-like growth factor I (LOC678666), mRNA	294	294	12%	3e-75	96%
Gallus gallus haplotype IGF-1-h8 insulin-like growth factor I (IGF-1) gene, complete cds	291	291	11%	4e-74	100%
Gallus gallus haplotype IGF-1-h7 insulin-like growth factor I (IGF-1) gene, complete cds	291	291	11%	4e-74	100%

Fig 2

It is evident from the sequence analysis of this amplicon of restricted fragment share its similarity with scaffold 27 of *Apteryx australis*(79% identity*), IGF-1* gene intron 1 and partial cds of *Anser anser* (79% identity) andIGF-1 Complete cds of *Gallus gallus* (97% identity).

Conclusion

It can be concluded that RFLP can be used as a powerful molecular technique for establishing the genetic diversity within a group. The RFLP pattern of PCR amplified DNA fragments from IGF1 gene region extracted from Red and Black Jungle fowl of Himalayan region revealed the typical band pattern. The generated RFLP pattern can be qualitatively identified or differentiated as per which samples 2, 3 and 7 are almost identical indicating them to be of same species. The sequence as obtained for the 9th sample show highest similarity with the fragments of IGF1 of *Apteryx australis, Anser anser* and *Gallus gallus* that is phylogenetically very close to the Indian chicken species.

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