RESEARCH ARTICLE

Comparative Genetic Characterization of *Prolactin* Gene between Giriraja and Indigenous Chicken

Ajeet Singh[1], Naveen Kumar, S.[1]*, Yathish, H.M.[1], Basavraj Inamdar[1], Malathi, V.[2]

Abstract

The present study was conducted to determine the polymorphism and to explore the possible SNPs in *prolactin* gene among the Giriraja and Indigenous chicken using PCR-RFLP. About 80 birds each of Giriraja and Indigenous chicken were genotyped for promoter regions 1 and 2 (C-2402T and C- 2161G) of prolactin gene by employing published primers. The restriction enzymes, *AluI* and *Csp6I* were utilized for genotyping polymorphisms of C-2402T and C-2161G, respectively. At promoter region 1 (24 bp indel at -358) of prolactin gene, the proportions of DD and ID genotypes were higher in Giriraja (0.45) and Indigenous (0.44) chicken. However, the frequency of D allele (0.775 and 0.631) was higher in both the studied populations. Whereas, for promoter region 2 of prolactin gene, the frequencies of T allele (C-2402T) and G allele (C-2161G) were higher in Giriraja (0.806 and 0.694) and Indigenous (0.644 and 0.525) confirming that T alleles are predominant in native or indigenous chicken. Further, three SNPs, *viz.,* C>T transition at 161 bp, T>C transition at 371 bp and C>G transversion at 402 bp were detected at promoter region 2 (C-2402T and C-2161G) in T/G allele compared to C allele.

Keywords: Broodiness, Giriraja, Indigenous chicken, Prolactin gene, Promoter.

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Introduction

Chicken are the most popular poultry worldwide irrespective of culture and region. Backyard farming has over the years contributed to a great extent to the agrarian economy of different countries. It provides livelihood security to the family in addition to securing the availability of food. Unemployed youth and women can also earn an income through backyard poultry farming. Indigenous breeds are well known for their tropical adaptability and disease resistance, while their plumage colour helps in protecting themselves against predators (Padhi, 2016). Many public sectors and few private sectors developed improved backyard varieties like Giriraja, Vanaraja, Gramapriya, Srinithi, etc., which are substantially contributing to the total chicken egg and meat production of the country (Chatterjee and Rajkumar, 2015). Giriraja is a dual-purpose bird developed by Karnataka Veterinary Animal and Fisheries Sciences University in Bangalore, India by crossing Red Cornish, White Cornish, White Rock and New Hampshire, which is suitable for both backyard rearing and intensive system of management. Giriraja females lay a large number of eggs (130-150 per year) with each egg weighing 52-55 grams. Their shells are brown in color and thicker than that of other commercial eggs and resist breaking. The birds exhibit better growth compared to local varieties and are suited for mixed and backyard farming (Somu, 2015).

Broodiness is a maternal behaviour in birds often known as incubation behaviour, which has been lost during domestication in some breeds of improved chicken, *viz.,* White Leghorn. Whereas many breeds of chicken especially unimproved ones including Red Jungle fowl have retained this behaviour with hens (Basheer *et al*., 2015). Polymorphism of prolactin (PRL) gene has been studied extensively in domestic chickens with regard to reproduction. Many researchers have associated Single Nucleotide Polymorphisms (SNPs) of PRL with egg production and broodiness (Kulibaba and Podstreshnyi, 2012; Ahmadi *et al.,* 2019), which infers that prolactin is important in the maintenance of broodiness. Hence the present study was taken up with the objectives to determine the polymorphism, identify the possible SNPs and compare the genetic variation in *prolactin* gene among Indigenous and Giriraja chicken.

[1]Department of Animal Genetics and Breeding, Veterinary College, KVAFSU, Hebbal, Bengaluru-560 024, Karnataka, India

[2]Department of Poultry Science, Veterinary College, KVAFSU, Hebbal, Bengaluru-560 024, Karnataka, India

Corresponding Author: Naveen Kumar, S., Department of Animal Genetics and Breeding, Veterinary College, KVAFSU, Hebbal, Bengaluru-560 024, Karnataka, India. e-mail: navingen@gmail.com

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Materials and Methods

Collection of Blood Samples and DNA Isolation

For the present study, approval of Institutional Animal Ethics Committee (IAEC) was obtained vide no. VCH/IAEC/2021/07

dated: 27.07.2021. Blood samples were collected from 80 Giriraja and 80 Indigenous birds maintained under Department of Poultry Science, Veterinary College, Bengaluru (India). About 1.5 mL of venous blood was collected aseptically by puncturing wing vein using a 24-gauge 1.5-inch hypodermic needle into a sterile vacutainer containing EDTA as an anticoagulant. The samples were transported to the laboratory in an icebox (4°C) and processed within 24 h. The conventional Phenol: Chloroform: Iso-amyl alcohol (25:24:1) method as described by Khosravinia *et al.* (2007) was employed for DNA extraction from whole avian blood.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was carried out by employing published primers. Promoter region 1 (130 or 154 bp) of *prolactin* gene containing the 24-bp indel at the site of −358 was amplified by forward primer: 5'-TTTAATATTGGTGGGTGAAGAGACA-3'; reverse primer: ATGCCACTGATCCTCGAAAACTC-3', whereas promoter region 2 (439 bp) of *prolactin* gene containing two SNPs, C-2402T and C-2161G was amplified by forward primer: 5'-AGAGGCAGCCCAGGCATTTTAC-3'; reverse primer: 5'-CCTGGGTCTGGTTTGGAAA TTG-3' (Bagheri Sarvestani *et al.,* 2013; Azhaguraja, 2017). PCR was performed in a final volume of 25 µL containing 12.5 µL of 2X Red PCR Master Mix, 1.5 µL (10 pmol/µL) each of forward and reverse primers, 1.0 µL of template DNA and 8.5 µL of PCR grade water. PCR was performed in Master cycler gradient (Bio Rad S1000, USA). For the amplification of promoter region 1, PCR reaction was carried out with an initial denaturation at 95°C (3 min), 35 cycles of 95°C (30 sec), 58°C (30 sec) and 72°C (30 sec) followed by a final extension at 72°C (5 min). Whereas, for amplification of promoter region 2 PCR reaction was carried out with an initial denaturation at 95°C (3 min), 35 cycles of 95°C (30 sec), 60°C (30 sec) and 72°C (30 sec) followed by a final extension at 72°C (5 min). The size of the PCR amplicons were confirmed by resolving on 1.5 % agarose gel along with 50 bp and 100 bp DNA ladders for promoter regions 1 and 2, respectively.

Restriction Fragment Length Polymorphism Analysis

The restriction enzymes (RE) *Alu*I and *Csp6*I were used to digest PCR amplicons of promoter region 2 of *Prolactin* gene for identification of C-2402T and C-2161G, respectively. The digestion was performed in a total volume of 30 µL which consisted of 10 µL of PCR product, 2 µL of 10X buffer, 0.5 µL (10U/µL) of RE and 17.5 µL of nuclease free water incubated at 37°C for 3 h followed by inactivation at 65°C for 20 min as per manufacturer's recommendations. The restriction enzyme digested PCR products were electrophoresed on 2.5 % agarose gel along with 50 bp DNA ladder. The genotype was determined by scoring the bands under the gel documentation system. The allele frequency, genotype frequency, and observed and expected heterozygosity were calculated as described by Rosner (2005).

Sequence Analysis

The PCR amplified products of promoter region 1 (24 bp indel at -358) of *Prolactin* gene and PCR amplified products of promoter region 2 (C-2402T and C-2161G) showing different patterns in RFLP were custom sequenced by double pass sequencing method using respective primers used for amplification of different products. The sequencing (double pass sanger sequencing method) was done at Barcode Biosciences, Bengaluru and the resultant sequences were analyzed using CLC Main Workbench software (CLC BIO, 2011).

Results and Discussion

The amplified product sizes of 130/154 bp (Plate 1) and 439 bp (Plate 2) were observed for promoter regions 1 (24 bp indel at -358) and 2 (C-2402T and C-2161G) of *prolactin (PRL)* gene, respectively, in Giriraja and Indigenous Chicken.

PCR Results

The PCR amplicon fragments of promoter region 1 (24 bp indel at -358) of *prolactin* gene resolved on 1.5 % agarose gel were scored and genotypes were identified. The samples with fragment size of 154 bp which included 24 bp insertion at the site -358 was considered as II genotype and the samples with fragment size of 130 bp which had 24 bp deletion at the site -358 was considered as DD genotype. Whereas, the samples with both fragments (154 bp and 130 bp) was considered as ID genotype. All the three genotypes were observed in Indigenous chicken whereas, only ID and DD genotypes were observed in Giriraja chicken (Plate 3). The allelic and genotypic frequencies, observed and expected heterozygosity, and Chi square values for promoter region 1 (24 bp indel at -358) of *prolactin* gene are presented in Table 1. In the present study, the frequencies of I and D alleles were 0.225 and 0.775, respectively, in Giriraja chicken and 0.369 and 0.631, respectively, in Indigenous chicken. The frequency of D allele was predominant in both the populations, which is in accordance with reports of Kulibaba and Podreshnyi

Table 1: Allelic and genotypic frequencies, observed and expected heterozygosity and χ^2 value for promoter region 1 (24 indel at -358 site) of *prolactin* gene

Breed/ Strain	Allelic frequency		Genotypic frequency			Observed heterozygosity (Ho)	Expected heterozygosity (He)	Chi square value
	I	D	II	ID	DD			
Giriraja chicken	0.225	0.775	0.00	0.45	0.55	0.45	0.35	-
Indigenous chicken	0.369	0.631	0.15	0.44	0.41	0.44	0.47	0.29^{NS}

Note: NS-non significant

(2012) in Ukranian meat-egg laying chicken (Line G-2) and Vinh *et al*. (2021) in Ri and Mia chicken of Vietnam. However, contrary to the present findings higher proportion of I allele was reported by Kulibaba and Podstreshnyi (2012) in Ukranian egg laying chicken, Rashidi *et al.* (2012) in Indigenous chicken of Mazandaran province of Iran, Bagheri Sarvestani *et al.* (2013) in Fars native chickens of Iran, Tempfli *et al.* (2015) in Hungarian Yellow chicken, Yadav *et al.* (2018) in Kadaknath chicken and Azhaguraja (2017) in Native chicken of Kerala. Further, Kulibaba and Podstreshnyi (2012) have concluded that I allele could be attributable for a better egg production. The observed and expected heterozygosities in the present study were 0.45 and 0.35, respectively, in Giriraja chicken and 0.44 and 0.47, respectively, in Indigenous chicken. These results were in concurrence with reports of Kulibaba and Podstreshnyi (2012) in Ukranian meat-egg and egg laying chicken. In the present study, the Indigenous chicken population was in Hardy Weinberg equilibrium indicating the randomness of the sample collected and absence of external forces, *viz*., selection, mutation and migration in the population, which is in agreement with Kulibaba and Podstreshnyi (2012), and Tempfli *et al*. (2015). However, the populations of Ukranian egg laying chicken (Kulibaba and Podstreshnyi, 2012) and Indigenous chicken of Mazandaran province of Iran (Rashidi *et al*., 2012) were reported to be deviated from equilibrium.

PCR-RFLP Results

PCR-RFLP analysis of promoter 2 region (C -2402T) of *prolactin* gene revealed two genotypes (CT and TT) in Giriraja and three genotypes (CC, CT and TT) in Indigenous chicken (Plates 4, 5). The allelic and genotypic frequencies, observed and expected heterozygosities, and Chi square values for promoter region 2 (C-2402T) of *prolactin* gene are presented in Table 2. In the present study, the allelic frequencies were 0.194 and 0.806 in Giriraja chicken and 0.356 and 0.644 in Indigenous chicken for C and T alleles, respectively. The frequency of T allele was higher in both the studied population. In concurrence to the present findings, higher frequency of T allele was reported by Kulibaba and Podstreshnyi (2012) in Ukrainian meat-egg laying chicken, Kulibaba *et al*. (2018) in Plymouth Rock White, Poltava clay and Rhode Island Red and by Vinh *et al.* (2021) in Ri and Mia chicken of Vietnam. Whereas, contrary to the present findings, higher frequency of C allele was reported by Kulibaba and Podstreshnyi (2012) in Ukrainian egg laying chicken (Line A), Rashidi *et al.* (2012) in Indigenous chicken of Mazandaran province of Iran, BagheriSarvestani *et al.* (2013) in Fars native chickens of Iran, Azhaguraja (2017) in IWN strain of White leghorn and native chicken of Kerala and by Kulibaba *et al.* (2018) in Borkovskaya Barvistaya. The frequency of allele C is higher in commercial lines of the White Leghorn, which reaches 1.00 (Manoharan *et al.,* 2020). Hence, higher frequency of C allele is often observed in egg laying type chicken (commercial lines) whereas, higher frequency of T allele is observed in indigenous/ native chicken with few exceptions. The observed and expected heterozygosities were 0.39 and 0.31, respectively, in Giriraja chicken and 0.56 and 0.46, respectively, in Indigenous chicken. Chi square test indicated that the Indigenous chicken population studied was in Hardy Weinberg equilibrium indicating the randomness of the sample collected and absence of external forces, *viz*., selection, mutation and migration in the population.

PCR-RFLP analysis of promoter 2 region (C-2161G) of *prolactin* gene revealed three genotypes (CC, CG and GG) in both Giriraja (Plate 6) and Indigenous chicken (Plate 7). The allelic and genotypic frequencies, observed and expected heterozygosities, and Chi square values for promoter region 2 (C-2161G) of *prolactin* gene are presented in Table 3. The frequencies of C and G alleles were 0.306 and 0.694, respectively, in Giriraja chicken and 0.475 and 0.525, respectively, in Indigenous chicken. In both the studied population, the frequency of G allele was higher.

Table 2: Allelic and genotypic frequencies, observed and expected heterozygosity and χ^2 value for promoter region 2 (C-2402T) of *prolactin* gene

Breed/ Strain	Allelic frequency		Genotypic frequency			Observed heterozygosity (Ho)	Expected heterozygosity (He)	Chi square value
	C	T	CC	CT	TT			
Giriraja chicken	0.194	0.806	0.00 (0)	0.39 (31)	0.61 (49)	0.39	0.31	-
Indigenous chicken	0.356	0.644	0.08 (6)	0.56 (45)	0.36 (29)	0.56	0.46	4.09^{NS}

Figures in the parenthesis indicate the number of birds; NS- non significant

Table 3: Allelic and genotypic frequencies, observed and expected heterozygosity and χ^2 value for promoter region 2 (C-2161G) of *prolactin* gene

Breed/ Strain	Allelic frequency		Genotypic frequency			Observed heterozygosity (Ho)	Expected heterozygosity (He)	Chi square value
	C	G	CC	CG	GG			
Giriraja chicken	0.306	0.694	0.10 (8)	0.41 (33)	0.49 (39)	0.41	0.42	0.068^{NS}
Indigenous chicken	0.475	0.525	0.15 (12)	0.65 (52)	0.20 (16)	0.65	0.50	7.36*

Figures in the parenthesis showing number of birds; *Significant at $p<0.05$

In contrast to the present findings, Azhaguraja (2017) reported presence of only C allele with frequency of 1.00 in IWN strain of White Leghorn. Further, Azhaguraja (2017) also reported higher frequeny of C alleles in Native chicken of Kerala. The differences observed between Indigenous chicken and Native chicken of Kerala may be attributed to unknown inclusion of exotic germplasm in the backyard of Kerala as reported by Manoharan *et al.* (2021). In the present study, the observed and expected heterozygosities were 0.41 and 0.42, and 0.65 and 0.50, respectively, in Giriraja chicken and Indigenous chicken. Further, Giriraja chicken population was in Hardy Weinberg equilibrium indicating the randomness of the sample collected and absence of external forces, *viz*., selection, mutation and migration in the population.

Sequence Analysis

The sequences of I and D alleles of promoter region 1 (24 bp indel at -358) of *prolactin* gene were subjected to alignment using CLC Main Workbench 6.8.1. which revealed 100 % identity of D alleles between Giriraja and Indigenous chicken. Further, a 24 bp insertion in I allele was observed when compared to D allele (Plate 8). Similar findings were reported by Azhaguraja (2017) and Manoharan *et al.*(2021).

For promoter region 2 (C-2402T and C-2161G) of the *prolactin* gene, multiple sequence alignment of C and T alleles (Plate 9) at C-2402T revealed 100 % identity between T allele sequences of Giriraja and Indigenous chicken and multiple sequence alignment of C and G alleles (Plate 11) at C-2161G revealed 100 % identity between C allele sequences of Giriraja and Indigenous chicken, and G allele sequences of Giriraja and Indigenous chicken. Further, C allele at C-2402T had 100 % identity with C allele at C-2161G and T allele at C-2402T had 100 % identity with G allele at C-2161G. Three SNPs, *viz*., C>T transition at 161 bp, T>C transition at 371 bp and C>G transversion at 402 bp (Plates 10 and 12) were detected in T/G allele of promoter region 2 (C-2402T and C-2161G) of *prolactin (PRL)* gene in both Giriraja and Indigenous chicken. Similar findings were reported by Azhaguraja (2017) in Native chicken of Kerala.

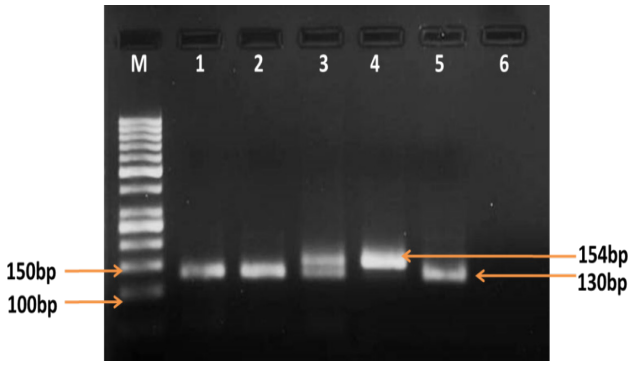

Plate 1: Agarose gel (1.5 %) picture showing PCR amplicons of promoter region 1 (24 bpindelat -358) of *prolactin* gene: Lane M: Molecular marker (50 bp ladder), Lanes 1, 2, 5: PCR amplified product of size 130 bp, Lane 3: PCR amplified product of size 130 bp and 154 bp, Lane 4: PCR amplified product of size 154 bp, and Lane 6: No Template Control

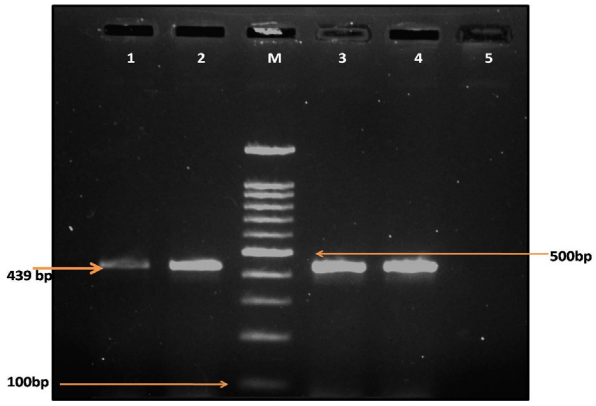

Plate 2: Agarose gel (1.5 %) picture showing PCR amplicons of promoter region 2 (C-2402T and C- 2161G) of *prolactin* gene: Lane M: Molecular marker (100 bp DNA ladder), Lanes 1, 2: PCR amplified product (439 bp) of Giriraja chicken, Lanes 3, 4: PCR amplified product (439 bp) of Indigenous chicken, and Lane 5: No Template Control.

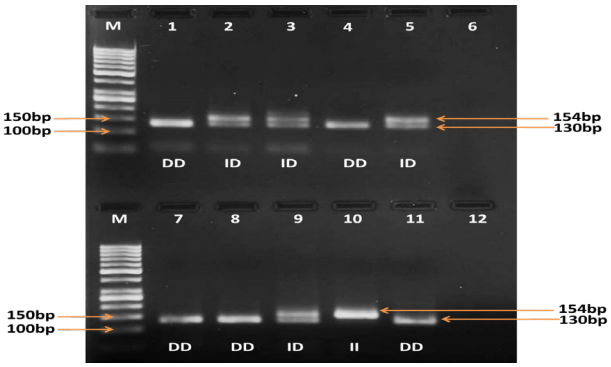

Plate 3: Agarose gel (1.5 %) picture showing PCR amplicons for different genotypes of promoter region 1 (24 bpindel at -358) of prolactin gene: Lane M: Molecular marker (50 bp ladder), Lanes 1, 4: PCR amplicon of 130 bp indicating DD genotype (Giriraja chicken), Lanes 2, 3, 5: PCR amplicon of 130/ 154 bp indicating ID genotype (Giriraja chicken), Lanes 7, 8, 11: PCR amplicon of 130 bp indicating DD genotype (Indigenous chicken), Lane 9: PCR amplicon of 130/ 154 bp indicating ID genotype (Indigenous chicken), Lane 10: PCR amplicon of 154 bp indicating II genotype (Indigenous chicken), and Lanes 6 and 12: No Template Control.

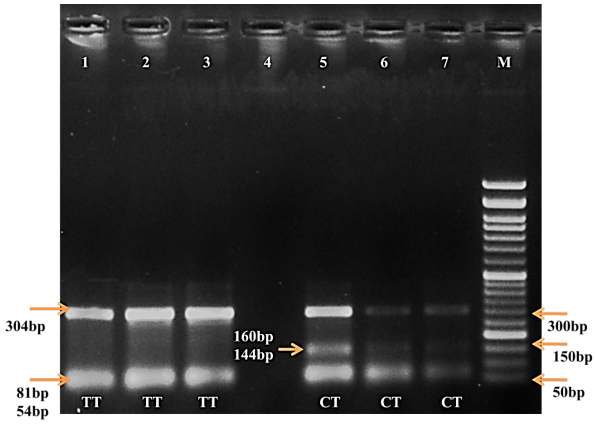

Plate 4: Agarose gel (2.5 %) picture PCR-RFLP patterns of promoter 2 region (C-2402T) of *prolactin* gene in Giriraja chicken: Lane M: Molecular marker (50 bp DNA ladder), Lanes 1, 2, 3: TT genotype (304 bp, 81 bp, 54 bp) and Lanes 5, 6, 7: CT genotype (304 bp, 160 bp, 144 bp, 81 bp, 54 bp)

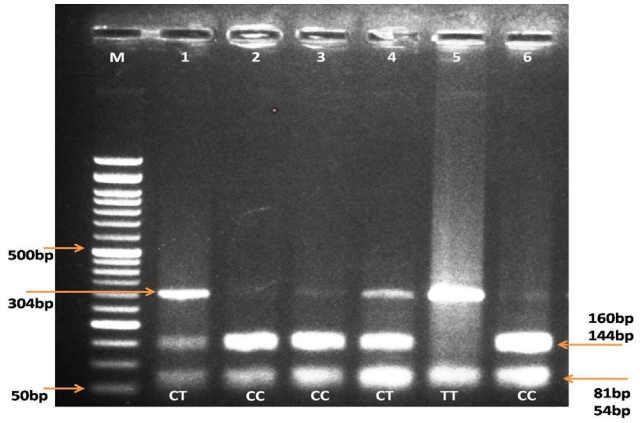

Plate 5: Agarose gel (2.5 %) picture PCR-RFLP patterns of promoter 2 region (C-2402T) of *prolactin* gene in Indigenous chicken: Lane M: Molecular marker (50 bp DNA ladder), Lanes 1, 4: CT genotype (304 bp, 160 bp, 144 bp, 81 bp, 54 bp), Lanes 2, 3, 6: CC genotype (160 bp, 144 bp, 81 bp, 54 bp) and Lanes 5: TT genotype (304 bp, 81 bp, 54 bp)

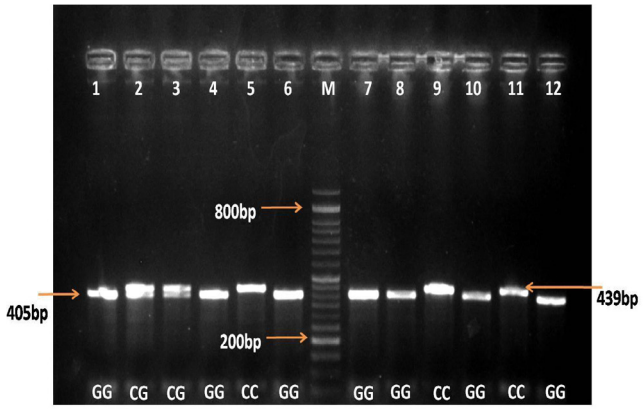

Plate 6: Agarose gel (2.5 %) picture PCR-RFLP patterns of promoter 2 region (C-2161G) of *prolactin* gene in Giriraja chicken: Lane M: Molecular marker (50 bp DNA ladder), Lanes 5, 9, 11: CC genotype (439 bp), Lanes 2, 3: CG genotype (439 bp, 405 bp) and Lanes 1, 4, 6, 7, 8, 10, 12: GG genotype (405 bp)

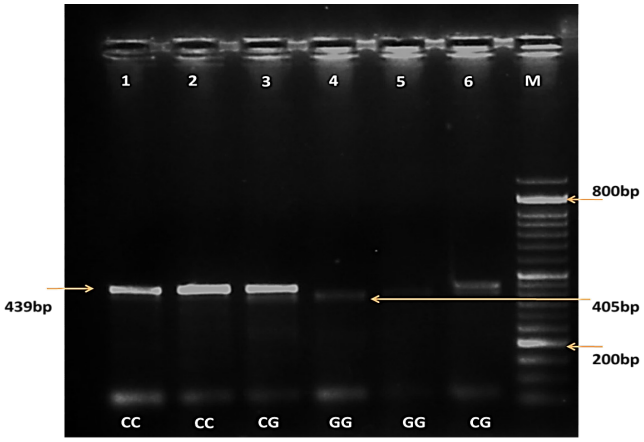

Plate 7: Agarose gel (2.5 %) picture PCR-RFLP patterns of promoter 2 region (C-2161G) of *prolactin* gene in Indigenous chicken: Lane M: Molecular marker (50 bp DNA ladder), Lanes 1, 2: CC genotype (439 bp), Lanes 3, 6: CG genotype (439 bp, 405 bp) and Lanes 4, 5: GG genotype (405 bp)

Conclusions

The present study demonstrated genetic variability in promoter region 1 (24 bp indel at -358) and promoter region 2 (C-2402T and C-2161G) of *prolactin (PRL)* gene in Giriraja and Indigenous chicken. The frequency of D allele of promoter region 1 (24 bp indel at -358) of *prolactin (PRL)* gene was predominant in both Giriraja and Indigenous chicken, which may indicate the presence of broodiness in these birds. However, further studies may be conducted to determine the association between D allele and broodiness. The proportion of T/G allele of promoter region 2 (C-2402T and C-2161G) of *prolactin (PRL)* gene was higher in both Giriraja and Indigenous chicken. This confirms that C alleles are predominant in egg laying commercial lines and T alleles are predominant in native or indigenous chicken.

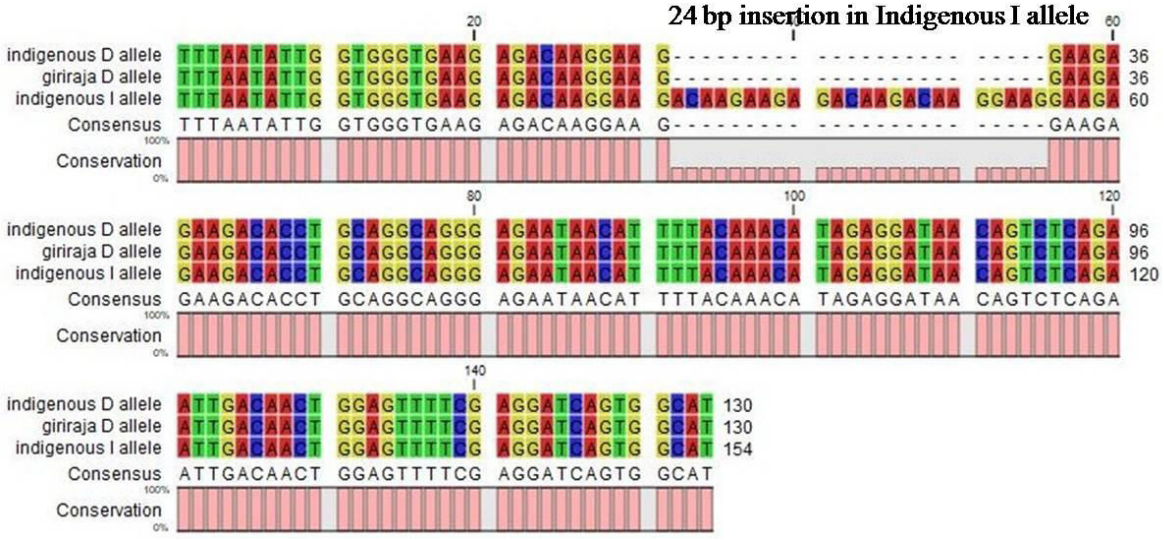

Plate 8: Alignment of I and D allele sequences of promoter region 1 (24 bpindel at the site of -358) of *prolactin* gene using CLC Main Workbench 6.8.1

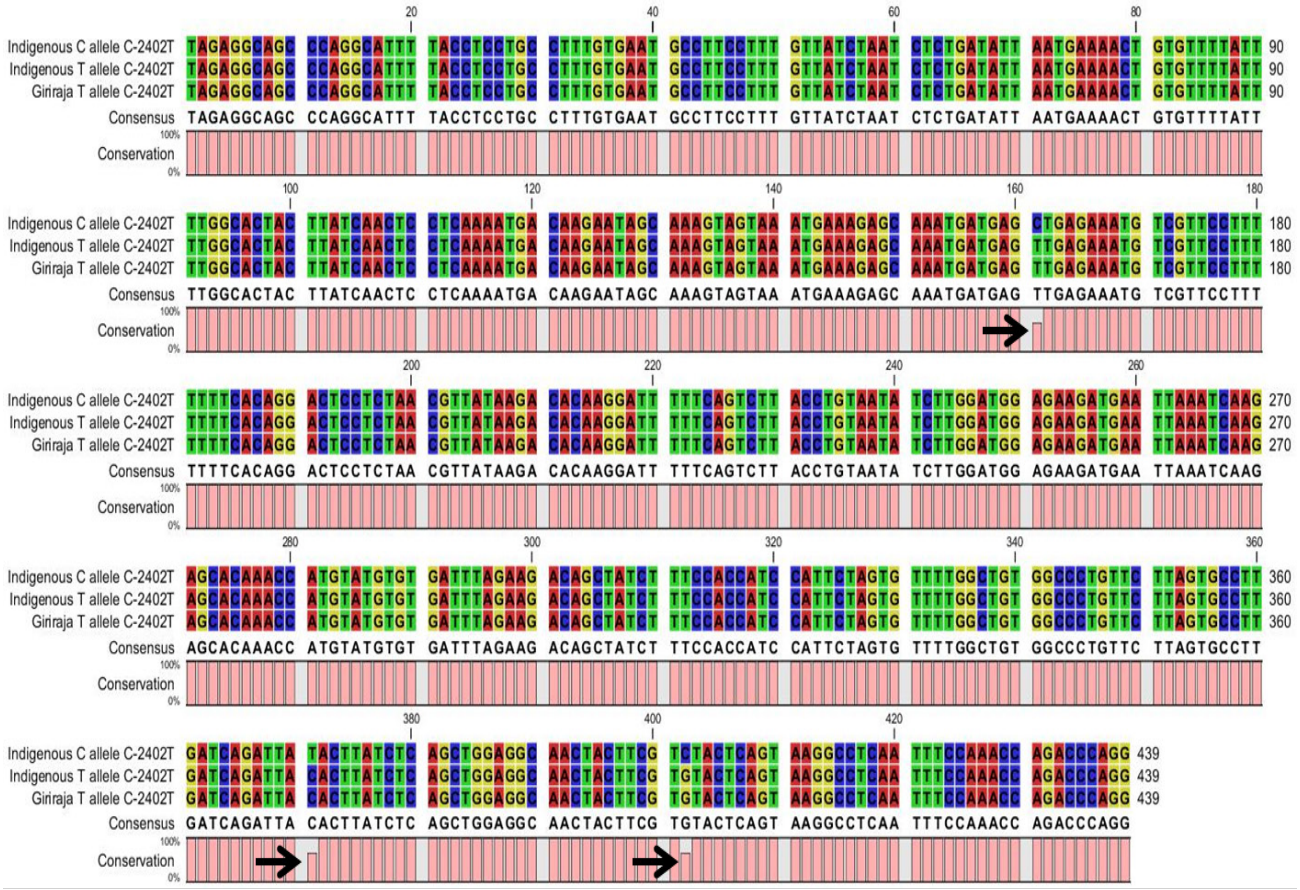

Plate 9: Nucleotide sequence alignment of C and T alleles of promoter region 2 (C-2402T) of *prolactin* gene using CLC Main Workbench 6.8.1

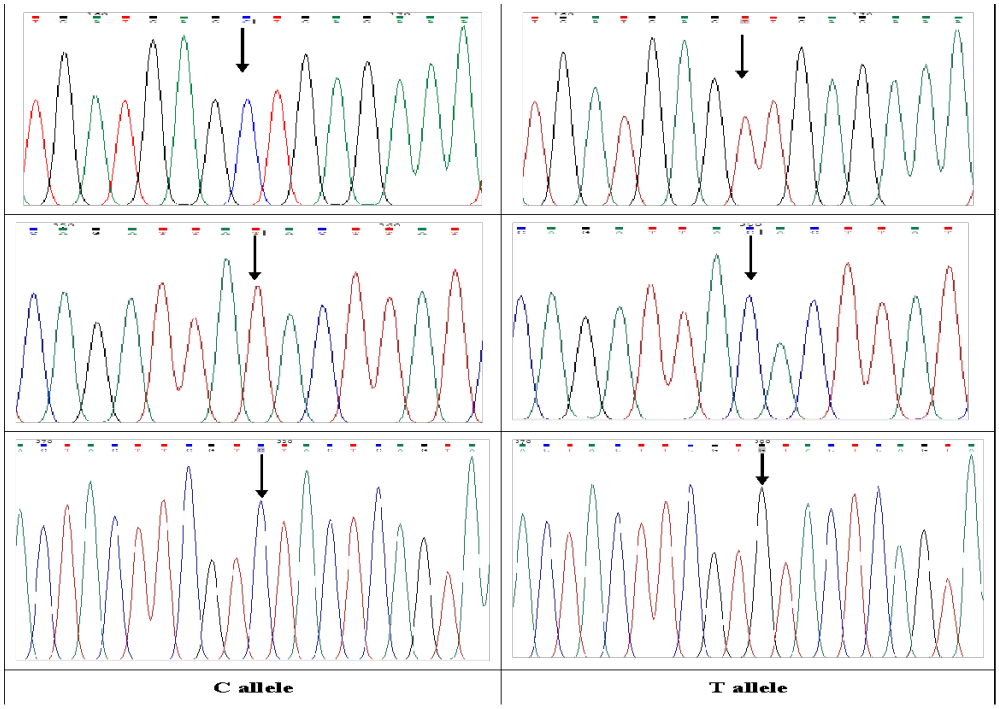

Plate 10: Chromatograms showing C>T transition at 161 bp, T>C transition at 371 bp and C>G Transversion at 402 bp in T allele of promoter region 2 (C-2402T) of *prolactin* gene

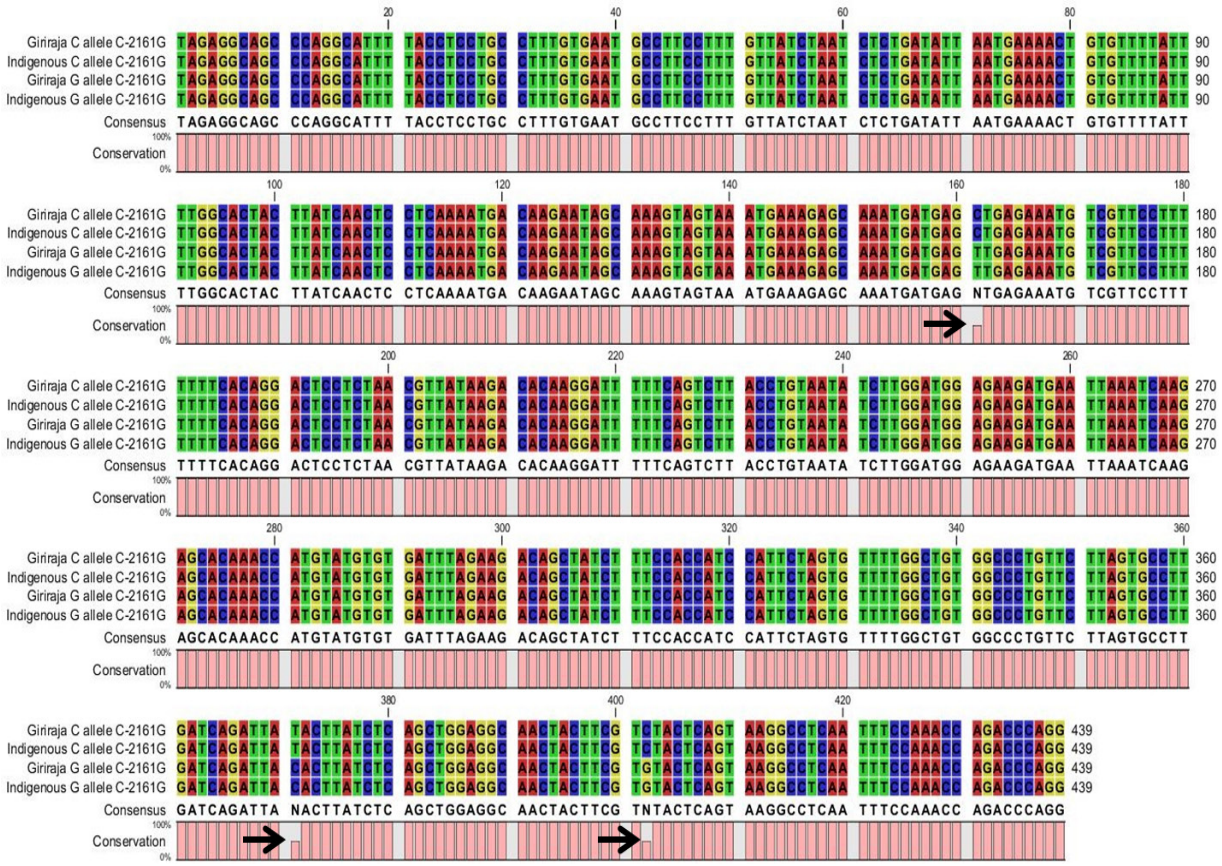

Plate 11: Nucleotide sequence alignment of C and G alleles of promoter region 2 (C-2161G) of *prolactin* gene using CLC Main Workbench 6.8.1

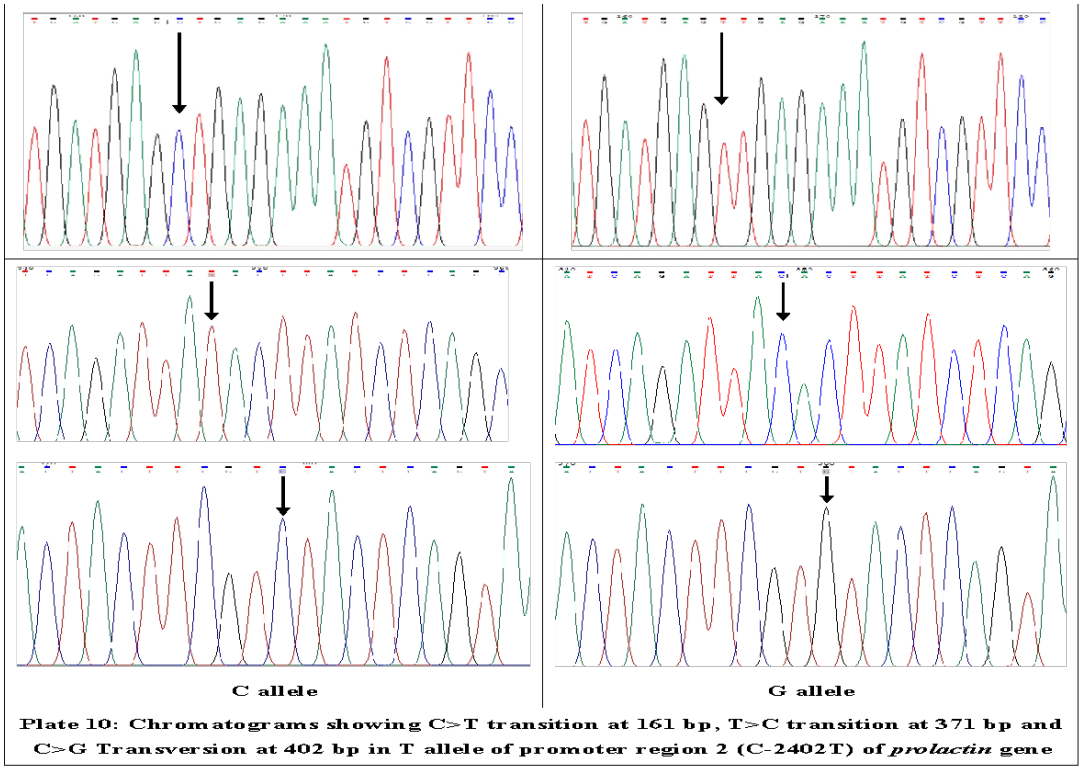

Plate 10: Chromatograms showing C>T transition at 161 bp, T>C transition at 371 bp and C>G Transversion at 402 bp in T allele of promoter region 2 (C-2402T) of *prolactin* gene

Plate 12: Chromatograms showing C>T transition at 161 bp, T>C transition at 371 bp and C>G Transversion at 402 bp in T allele of promoter region 2 (C-2161G) of *prolactin* gene

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