RESEARCH ARTICLE

Genetic Polymorphism of *NRAMP*-1 and *iNOS* Genes in Giriraja and Indigenous Chicken

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Abstract

The present study was conducted to identify and compare the genetic polymorphisms of Natural Resistance Associated Macrophage Proteins-1(*NRAMP*-1) and Inducible Nitric Oxide Synthase (*iNOS*) genes among Giriraja and Indigenous chicken. Blood samples were collected from 100 each of Giriraja and Indigenous chicken and the DNA was isolated by Phenol: Chloroform: Iso-amyl alcohol (25:24:1) method. Published primers were employed to amplify *NRAMP*-1 (exon 11) and *iNOS* (intron 24) genes. RFLP analyses of *NRAMP*-1 and *iNOS* genes were conducted utilizing *Sac*I and *Alu*I restriction enzymes, respectively. For *NRAMP*-1, the frequencies of TT, TC and CC genotypes were 0.16, 0.47 and 0.37, respectively, in Giriraja and 0.10, 0.57 and 0.33, respectively, in Indigenous chicken. The frequencies of T and C alleles were 0.395 and 0.605, in Giriraja and 0.385 and 0.615, respectively, in Indigenous chicken. For *iNOS*, the frequencies of TT, TC and CC genotypes were 0.26, 0.36 and 0.38, respectively, in Giriraja and 0.20, 0.53 and 0.27, respectively, in Indigenous chicken. The frequencies of T and C alleles were 0.440 and 0.560 in Giriraja and 0.465 and 0.535, respectively, in Indigenous chicken. The present study detected 5 SNPs (T>C transition at 128 bp; T>G transversions at 140 bp and 141 bp; T>C transition at 189 bp; A>G transition at 393 bp) in C allele of *NRAMP*-1 gene of Giriraja compared to Indigenous chicken and 2 SNPs (G>A transition at 107 bp and T>C transition at 130 bp) in C allele compared to T allele of *iNOS* gene in both Giriraja and Indigenous chicken.

Key words: Chicken, Disease resistance, Genetic polymorphism, *iNOS*, *NRAMP-1*, PCR-RFLP.

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Introduction

Backyard poultry farming is a powerful traditional practice for improvement of socio-economic status of rural poor with low-cost initial investment, high economic return and guarantee for improving protein deficiency among the poor. Backyard poultry production mainly comprises of rearing indigenous chicken. As the production performance of indigenous chicken is poor, improved varieties like Vanaraja, Gramapriya, Srinidhi, Giriraja etc. developed mostly by public sector (Chatterjee and Rajkumar, 2015) are substantially contributing to the backyard poultry population of the country. Poultry diseases, including those caused by viral, bacterial and parasitic infections can lead to substantial economic losses. Among the different diseases occurring in poultry, those caused by the genus *Salmonella* is the most common, causing serious economic losses to the poultry industry in terms of mortality, reduced growth and loss of egg production (Rychlik *et al*., 2014). *Salmonella* can be transmitted horizontally within the flock after fecal shedding as well as vertically through the trans-ovarian route. The level of bacterial colonization is under genetic control in either the young or adult avian host (Sadeyen *et al*., 2004).

Several research studies have reported that the Natural Resistance Associated Macrophage Proteins-1(*NRAMP-1*) and Inducible Nitric Oxide Synthase (*iNOS*) genes are associated with chicken resistance to *S. enteritidis* (Liu *et al.,* 2003; Malek and Lamont, 2003; Tohidi *et al.,* 2013). *NRAMP-1* gene belongs to a large gene family encoding divalent cation transporters that are localized to late endosomes/ lysosomes and are proposed to affect intra phagosomal microbial replication by modulating divalent cation content in this organelle (Liu *et al*., 2003). Many cellular functions that depend on metal ions as cofactors may explain the pleiotropic effects of *NRAMP-1* and its complex role in infectious diseases (Blackwell *et al*., 2000). About eleven sequence variations within the *NRAMP-1*

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gene have been identified that are closely associated with the differences in resistance to salmonellosis (Hu *et al*., 1997). *iNOS* is a cytosolic enzyme that catalyses the conversion of L-arginine to nitric oxide, a cytotoxic compound for pathogens in macrophages (Alderton *et al.,* 2001). Nitric oxide plays a powerful role in immune responses because of its antimicrobial and antitumor functions (Blanchette *et al*., 2003). *iNOS* has been identified as a candidate gene that controls resistance to *Salmonella enteritidis* in poultry (Kramer *et al*., 2003; Malek and Lamont, 2003). Giriraja and Indigenous chicken are acclaimed for their disease resistance. However, there is dearth of information regarding the genetic polymorphism of *NRAMP-1* and *iNOS* genes, which are two important genes associated with disease resistance, especially against salmonellosis. Therefore, the present study was undertaken to identify and compare the genetic polymorphisms and also to explore the possible SNPs in *NRAMP-1* and *iNOS* genes among Giriraja and Indigenous chicken.

Materials and Methods

The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) vide no.VCH/IAEC/2022/03, dated: 08.06.2022.

Blood Collection and Isolation of Genomic DNA

Two hundred random blood samples were collected from Giriraja (100) and Indigenous (100) chicken maintained under Department of Poultry Science, Veterinary College, KVAFSU, Hebbal, Bengaluru. The conventional Phenol: Chloroform: Iso-amyl alcohol (25:24:1) method for DNA extraction from whole avian blood as described by Khosravinia *et al*. (2007) was followed with few modifications. The purity and yield of genomic DNA were ascertained by spectrophotometer and 0.8 % agarose gel electrophoresis.

Polymerase Chain Reaction

Published primers, *viz*., Forward: 5'CAATGAGACGGTGTCTGTGG 3' and Reverse: 5' CCCAGAAGAAATCTCCCTGC 3' were employed to amplify exon 11 region of *NRAMP*-1 (Muhsinin *et al.,* 2016; Ardiyana *et al.,* 2020). The amplification was performed in a total volume of 25 µL consisting of 12.5 µL of 2X Red PCR Master Mix, 1 µL each of forward and reverse primer (10 pmol/ µL), 1 µL of template DNA and 9.5 µL of PCR grade water. The PCR reaction was carried out with an initial denaturation temperature of 95 °C (5 min), 35 cycles of 95 °C (10 sec), 62 °C (20 sec) and 72 °C (30 sec), followed by a final extension at 72 °C (5 min).

Amplification of intron 24 region of *iNOS* gene was done by employing published primers, *viz*., Forward: 5'CCAAGGACTTACAGGTGTGG 3' and Reverse: R: 5'CCAGGATGTTTGGGCTGTTG 3' (Muhammad *et al.,* 2018; Ardiyana *et al.,* 2020). The amplification was performed in a total volume of 25 µL consisting of 12.5 µL of 2X Red PCR Master Mix, 0.5 µL each of forward and reverse primer (10 pmol/ µL), 0.5 µL of template DNA and 10.5 µL of PCR grade water. The PCR reaction was carried out with an initial denaturation temperature of 95 °C (5 min), 35 cycles of 95 °C (10 sec), 56.3 °C (20 sec) and 72 °C (30 sec), followed by a final extension at 72 °C (5 min).

The PCR amplicons were confirmed by resolving on 1.5 % agarose gel along with 100 bp ladder at a constant voltage of 150 V for 45 to 60 minutes in 1X TAE buffer.

Restriction Fragment Length Polymorphism Analysis

Restriction enzymes namely, *Sac*I and *Alu*I were used to digest PCR amplicons of *NRAMP-1* and *iNOS* genes, respectively. For both the enzymes, digestion was done in a total volume of 30 µL which consisted of 2 µL of 10X buffer, 10 µL of PCR amplicon, 0.5 µL of RE, 17.5 µL of nuclease-free water (NFW) with incubation at 37 °C for 16 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 in parallel with 100 bp DNA ladder to visualize bands. Genotypes were determined based on visualization of different band patterns. The allelic frequency, genotypic frequency, and observed and expected heterozygosities were calculated.

Sequence Analysis

The PCR amplified products of *NRAMP-1* and *iNOS* genes showing different patterns in RFLP were custom sequenced by double pass sequencing method using respective primers used for amplification. The sequencing was done at Barcode Biosciences, Bengaluru and the resultant sequences were analyzed using CLC Main Workbench software (CLC BIO, 2011).

Results And Discussion

Isolation of Genomic DNA

The Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) method for isolation of genomic DNA from venous blood yielded a good amount of DNA. The ratio of spectrophotometer absorbance readings at 260 and 280 nm for isolated DNA samples were in the range of 1.7 to 2.0, indicative of good purity of genomic DNA. A good concentration of 50 to150 µg DNA was isolated from 50 µL of blood samples. Similar yield was obtained by Ramesh *et al*. (2022) in the same laboratory. However, Harish Kumar (2016) reported 100 µg of DNA from 50 µL of blood and Ajeet Singh (2022) reported 50-200 µg of genomic DNA from 80 µL of blood samples. Majority of the genomic DNA samples showed clear and distinct bands on 0.8 % Agarose gel electrophoresis, inferring high quality DNA (Plate 1).

PCR-RFLP Analysis

All the samples of Giriraja and Indigenous chicken yielded a single amplified product of 421 and 449 base pairs on 1.5 % agarose gel electrophoresis for *NRAMP-1* and *iNOS* genes, respectively (Plates 2 and 3).

For *NRAMP-1* gene, three different RFLP patterns were observed in both Giriraja and Indigenous chicken. Single fragment of 421 bp was identified as TT genotype, two fragments of 258 bp and 163 bp were identified as CC genotype, and three fragments of 421 bp, 258 bp and 163 bp were identified as TC genotype (Plate 4). Similar results were reported by Muhsinin *et al.* (2016), Hadrawi *et al.* (2016) and Ardiyana *et al.* (2020) in different breeds of chicken.

Plate 1: Agarose gel (0.8 %) picture showing genomic DNA
Lanes 1, 2, 3 and 4: Genomic DNA of Giriraja chicken
Lanes 5, 6, 7 and 8: Genomic DNA of Indigenous chicken

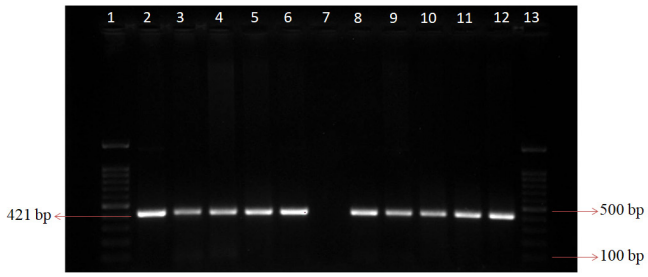

Plate 2: Agarose gel (1.5%) picture showing PCR amplicons of *NRAMP-1* gene
Lanes 1 and 13: 100 bp DNA ladder, Lanes 2, 3, 4, 5 and 6: PCR amplicons of Giriraja chicken
Lane 7: No Template Control and Lanes 8, 9, 10, 11 and 12: PCR amplicons of Indigenous chicken.

Similarly for *iNOS* gene, three different RFLP patterns were observed in both Giriraja and indigenous chicken. Fragment size of 449 bp was termed as TT genotype, fragments of sizes 310 bp and 139 bp were termed as CC genotype, and fragments of sizes 449 bp, 310 bp and 139 bp were termed as TC genotype (Plate 5). These results were in concurrence with the reports of Muhammad *et al.* (2018) and Ardiyana *et al.* (2020).

For *NRAMP-1* gene, the frequencies of TT, TC and CC genotypes were 0.16, 0.47 and 0.37, respectively, in Giriraja chicken and 0.10, 0.57 and 0.33, respectively, in indigenous chicken, whereas the frequencies of T and C alleles were 0.395 and 0.605, respectively, in Giriraja chicken and 0.385 and 0.615, respectively, in indigenous chicken (Table 1). In both the studied populations, C allele and the heterozygous, TC genotype were found to be predominant.

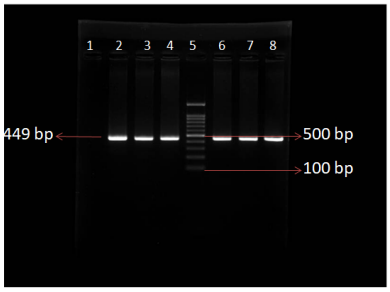

Plate 3: Agarose gel (1.5%) picture showing PCR amplicons of *iNOS* gene
Lane 1: No template control, Lanes 2, 3 and 4: PCR amplicons of Giriraja chicken,
Lane 5: 100 bp DNA ladder and Lanes 6, 7 and 8: PCR amplicons of Indigenous chicken.

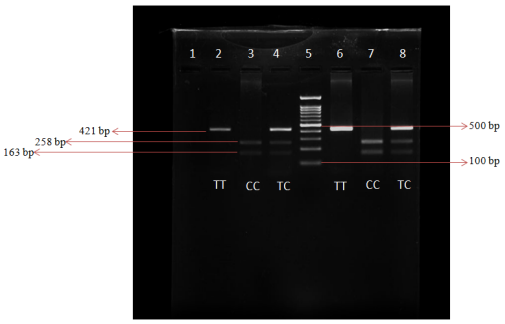

Plate 4: Agarose gel (2.5%) picture showing PCR-RFLP patterns of *NRAMP-1* gene
Lane 1: No template control, Lanes 2 to 4: PCR-RFLP patterns in Giriraja chicken,
Lane 5: 100 bp DNA ladder and Lanes 6 to 8: PCR-RFLP patterns in Indigenous chicken.

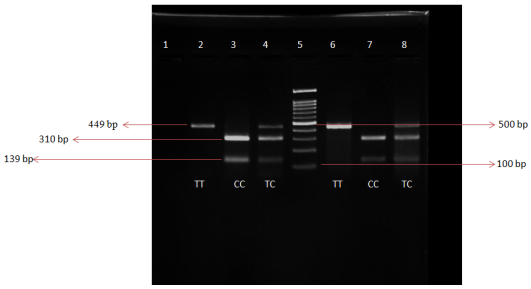

Plate 5: Agarose gel (2.5%) picture showing PCR-RFLP patterns of *iNOS* gene
Lane 1: No template control, Lanes 2 to 4: PCR-RFLP patterns in Giriraja chicken,
Lane 5: 100 bp DNA ladder and Lanes 6 to 8: PCR-RFLP patterns in Indigenous chicken

In agreement with the present findings, higher proportions of TC genotype and C allele were reported by Tohidi *et al.* (2011) in Malaysian native chickens. Similarly, predominance of C allele was also reported by Hadrawi *et al.* (2016) in Kampung chicken, Muhsinin *et al.* (2016) in Indonesian native

chicken and Ardiyana *et al.* (2020) in SenSi-1 Agrinak chicken of Indonesia. However, contrary to the present results, higher frequency of CC genotype was observed by Tohidi *et al.* (2013) in two native breeds of Malaysian chicken, Hadrawi *et al.* (2016) in Kampung chicken, Muhsinin *et al.* (2016) in Indonesian native chicken and Ardiyana *et al.* (2020) in SenSi-1 Agrinak chicken of Indonesia. Further, only two genotypes, CC and CT were observed in Kampung chicken, broilers, F1 Kampung X Broiler (KB) and F2 KB X KB (Muhsinin *et al.,* 2016). These differences may be attributed to the genetic makeup, breeds, the breeding programmes followed and the sample size of the birds.

The observed and expected heterozygosities in the present study were 0.470 and 0.478, respectively, in Giriraja chicken and 0.570 and 0.474, respectively, in Indigenous chicken (Table 1). The observed and expected heterozygosities were almost similar in both the populations indicating absence of imbalances in the population analyzed (Harbison and Nguyen, 2017). Moderate amount of heterozygosity revealed presence of moderate genetic variability in the population and indicated moderate survival of the population.

In the present study, Chi square test revealed that only Giriraja chicken population was in Hardy Weinberg equilibrium (Table 1), indicating the randomness of the sample collected and absence of external forces, *viz*., selection, mutation and migration in the population. The result is in concurrence with the reports of Ardiyana *et al.* (2020) in SenSi-1 Agrinak Chicken. Whereas, studied Indigenous chicken population was deviated from the Hardy Weinberg equilibrium which is in agreement with reports of Tohidi *et al.* (2011) in Malaysian native chickens and Hadrawi *et al.* (2016) in Kampung Chicken. These differences observed may be attributable to the breeds of chicken studied, intensity of selection, selection pressure and other forces acting on the population.

For intron 24 region of *iNOS* gene also, both the studied populations of Giriraja and Indigenous chicken revealed three genotypes namely, TT, TC and CC indicating polymorphism. The genotypic frequencies for TT, TC and CC were 0.26, 0.36 and 0.38, respectively, in Giriraja chicken, and 0.20, 0.53 and 0.27, respectively, in Indigenous chicken. The allelic frequencies for T and C were 0.440 and 0.560, in Giriraja chicken and 0.465 and 0.535, respectively, in indigenous chicken (Table 2). In Giriraja chicken, CC genotype was predominant, whereas TC genotype was predominant in Indigenous chicken population. However, C allele was predominant in both the studied populations.

In concurrence to the present findings, higher frequency of CC genotype was observed in Sentul chicken of Indonesia (Muhammad *et al.,* 2018) and higher proportion of TC genotype was observed in Malaysian native chickens (Tohidi *et al.,* 2011) and SenSi-1 Agrinak chicken of Indonesia (Ardiyana *et al.,* 2020). Moreover, predominance of C allele was reported by Tohidi *et al.* (2011) in Malaysian native chickens, Muhammad *et al.* (2018) in Sentul chicken of Indonesia and Ardiyana *et al.* (2020) in SenSi-1 Agrinak chicken of Indonesia. These differences may be attributed to the genetic makeup, breeds, the breeding programme followed and the sample size of the birds.

In the present study, the observed and expected heterozygosities were 0.360 and 0.493, respectively, in Giriraja chicken and 0.530 and 0.498, respectively, in Indigenous chicken (Table 2). Indigenous chicken population had higher heterozygosity indicating presence of greater variability in the population and absence of inbreeding. However, Giriraja population had lower heterozygosity inferring presence of inbreeding and differences in observed and expected heterozygosities indicated genetic imbalances existing in the population (Harbison and Nguyen, 2017).

Table 1: Allelic and genotypic frequencies, observed and expected heterozygosities and χ^2 value for *NRAMP-1* gene

Breed/ Strain	Allelic frequency		Genotypic frequency			Observed heterozygosity (Ho)	Expected heterozygosity (He)	Chi- square value
	T	C	TT	TC	CC			
Giriraja chicken	0.395	0.605	0.16 (16)	0.47 (47)	0.37 (37)	0.470	0.478	0.028^{ns}
Indigenous chicken	0.385	0.615	0.10 (10)	0.57 (57)	0.33 (33)	0.570	0.474	4.418*

Note: Figures in parenthesis indicate the number of animals; ns non-significant; *Significant at P<0.05.

Table 2: Allelic and genotypic frequencies, observed and expected heterozygosities and χ^2 value for *iNOS* gene

Breed/ Strain	Allelic frequency		Genotypic frequency			Observed heterozygosity (Ho)	Expected heterozygosity (He)	Chi square value
	T	C	TT	TC	CC			
Giriraja chicken	0.440	0.560	0.26 (26)	0.36 (36)	0.38 (38)	0.360	0.493	7.262**
Indigenous chicken	0.465	0.535	0.20 (20)	0.53 (53)	0.27 (27)	0.530	0.498	0.425^{ns}

Note: Figures in parenthesis indicate the number of animals; ns non-significant; **Significant at P<0.01.

Similarly higher degrees of observed and expected heterozygosities indicating greater variability were reported by Tohidi *et al*. (2011) in Malaysian native chickens (0.51 and 0.43) and Ardiyana *et al*. (2020) in SenSi-1 Agrinak chicken of Indonesia (0.773 and 0.496). Level of heterozygosity in the population can be used as an indication of the degree of endogamy (inbreeding) as a result of an intensive selection process (Machado *et al*., 2003; Ryan and Ray, 2014).

The studied indigenous chicken population was found to be in Hardy Weinberg equilibrium indicating the randomness of the sample and absence of external forces, *viz*., selection, mutation and migration in the population. This finding is in agreement with reports of Tohidi *et al*. (2011) in Malaysian native chicken and Tohidi *et al*. (2012) in Village chicken and Red Jungle fowl. However, Giriraja chicken population in the present study was deviated from Hardy Weinberg equilibrium (Table 2), which is in concurrence with the reports of Ardiyana *et al*. (2020) in SenSi-1 Agrinak Chickens. These differences observed may be attributed to the breeds of chicken studied, intensity of selection, selection pressure and other forces acting on the population.

Sequence Analysis

Representative samples of TT and CC genotypes of exon 11 region of *NRAMP-1* gene and intron 24 region of *iNOS* gene were selected randomly and were custom sequenced by double pass sequencing method. Sequencing confirmed the expected size of 421 and 449 base pairs and comparison of forward sequence with that of the reverse complement showed 100 % alignment, which indicated the correctness of the sequence.

For exon 11 region of *NRAMP-1* gene, multiple sequence alignment of T and C allele sequences of Giriraja and Indigenous chicken revealed 100 % identity (Plate 6) between T allele sequences of Giriraja and Indigenous chicken. However, 5 SNP's, *viz*., T>C transition at 128 bp; T>G transversions at 140 bp and 141bp; T>C transition at 189 bp; A>G transition at 393 bp were observed in C allele of Giriraja compared to Indigenous chicken (Plates 6 and 7).

With respect to intron 24 region of *iNOS* gene, multiple sequence alignment of T and C allele sequences of Giriraja and Indigenous chicken (Plate 8) revealed 100 % identity between T and C allele sequences of Giriraja and Indigenous chicken. However, apart from the restriction site 2 SNP's namely, G>A transition at 107 bp and T>C transition at 130 bp were observed in C allele compared to T allele (Plates 8 and 9). There were no earlier reports available with regard to sequence analysis of exon 11 region of *NRAMP-1* and intron 24 region of *iNOS* genes for comparison.

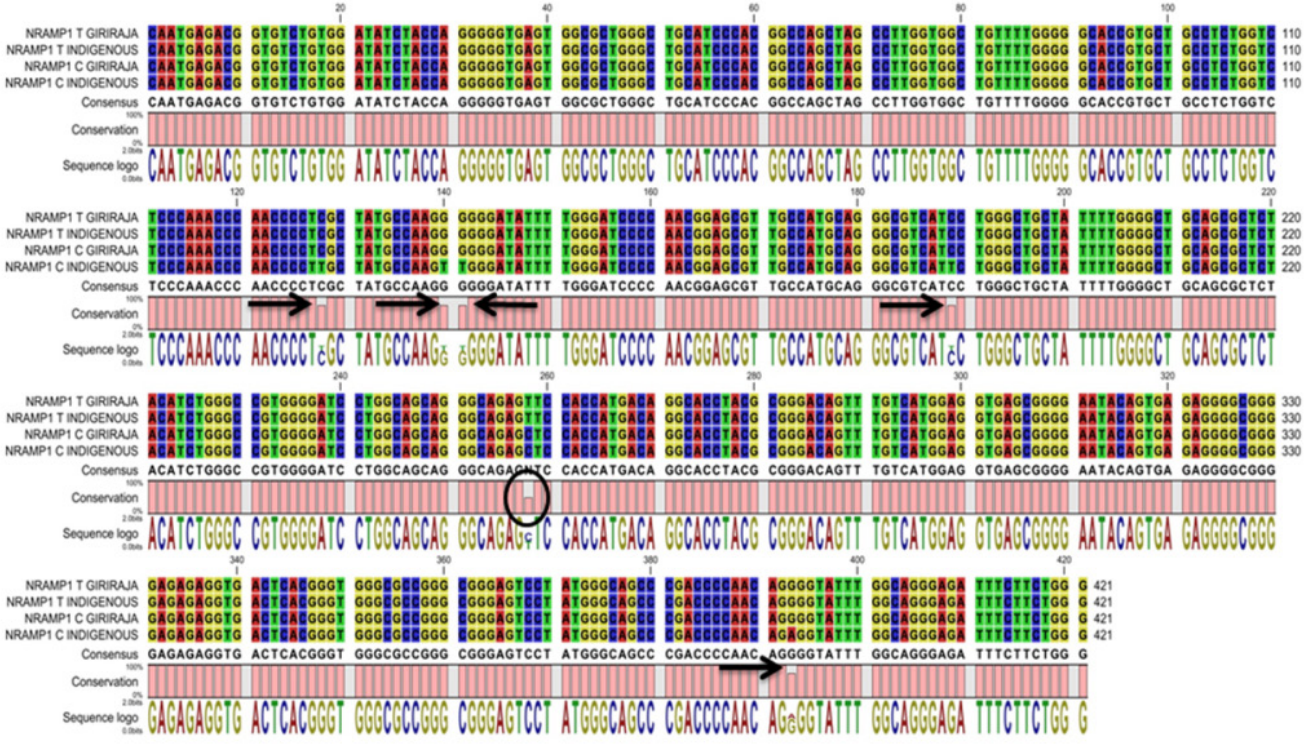

Plate 6: Nucleotide sequence alignment of T and C alleles of *NRAMP-1*gene using CLC Main Workbench 6.6.2

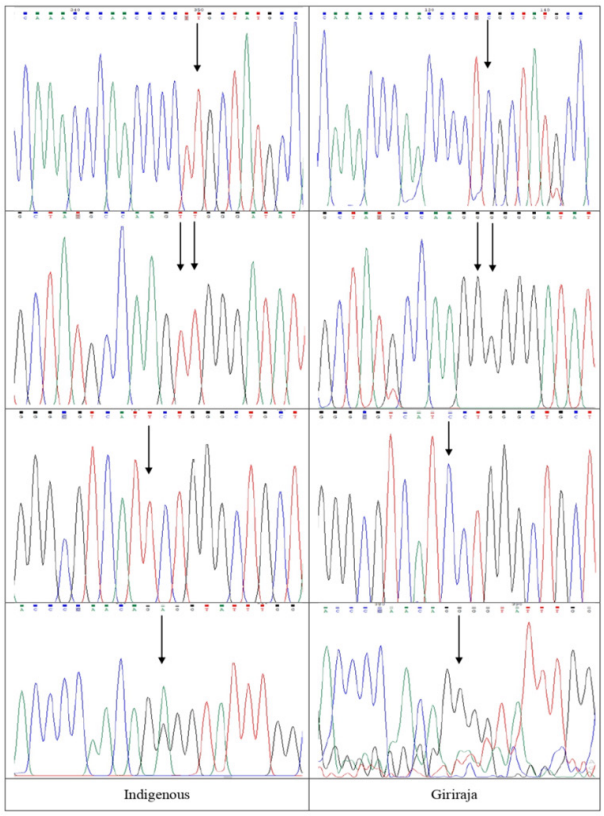

Plate 7: Chromatograms showing T>C transition at 128 bp; T>G transversions at 140 bp and 141 bp; T>C transition at 189 bp; A>G transition at 393 bp in C allele of *NRAMP-1* gene of Giriraja compared to Indigenous birds.

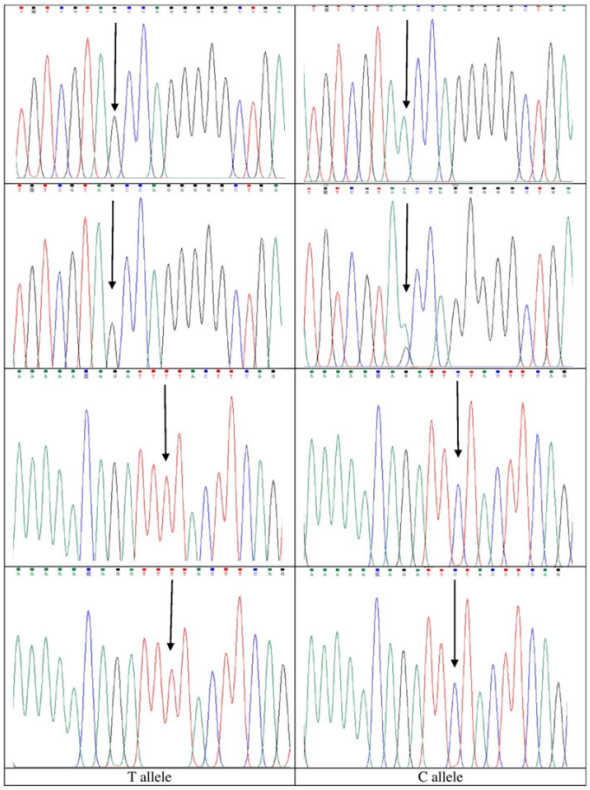

Plate 9: Chromatograms showing G>A transition at 107 bp and T>C transition at 130 bp in C allele compared to T allele of iNOS gene

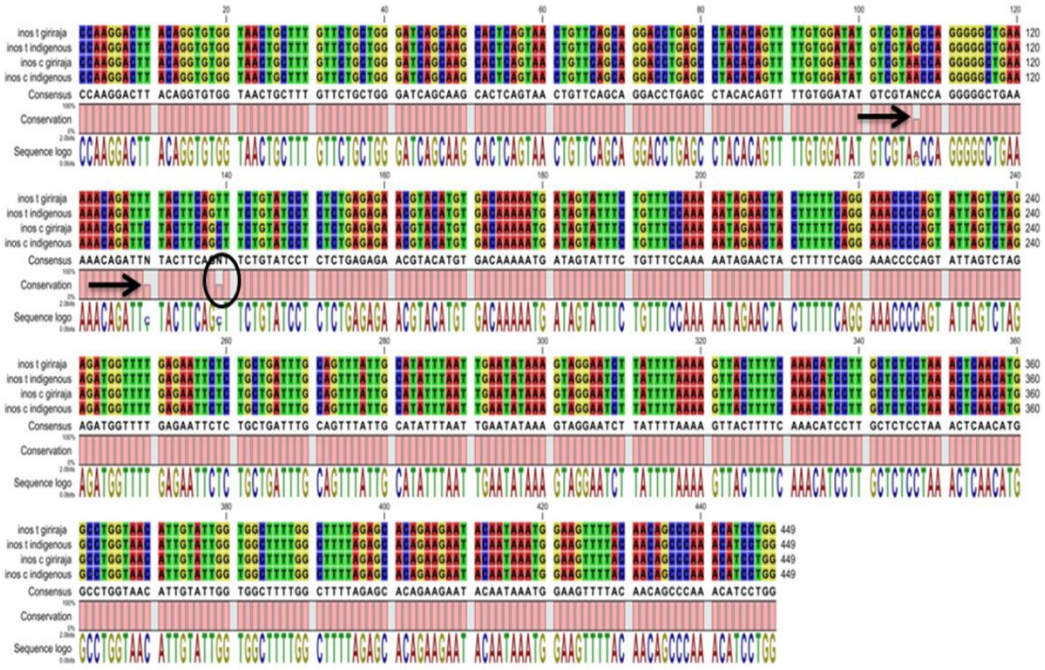

Plate 8: Nucleotide sequence alignment of T and C alleles of iNOSgene using CLC Main Workbench 6.6.2

Conclusions

The present study revealed polymorphisms of *NRAMP-1* (exon 11) and *iNOS* (intron 24) genes in Giriraja and Indigenous chicken by PCR-RFLP technique. Five novel SNPs, *viz*., T>C transition at 128 bp; T>G transversions at 140 bp and 141 bp; T>C transition at 189 bp; A>G transition at 393 bp were detected in C allele of *NRAMP-1* (exon 11) gene of Giriraja compared to Indigenous birds. With regard to *iNOS* (intron 24), apart from the restriction site, two novel SNPs namely, G>A transition at 107 bp and T>C transition at 130 bp were detected in C allele as compared to T allele in both Giriraja and Indigenous chicken. Further studies may be conducted to establish association between *NRAMP-1* and *iNOS* gene variants and disease resistance traits.

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References

Alderton, W.K., Cooper, C.E., & Knowles, R.G. (2001). Nitric oxide synthases: structure, function, and inhibition. *Biochemical Journal*, *357*: 593-615.

Ajeet Singh (2022). Comparative genetic characterization of *prolactin* gene between Giriraja and Indigenous chicken. *M.V.Sc. thesis*, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, India.

Ardiyana, M., Gunawan, A., Murtini, S., Sartika, T., & Sumantri, C. (2020). Polymorphisms and associations of the NRAMP-1 and iNOS genes on Newcastle disease and *Salmonella enteritidis* resistances in sensi-1 Agrinak Chickens. *Tropical Animal Science Journal*, *43*(2), 95-102.

Blackwell, J.M., Searle, S., Goswami, T., & Miller, E.N. (2000). Understanding the multiple functions of Nramp1. *Microbes and Infection*, *2*(3), 317-321.

Blanchette, J., Jaramillo, M., & Olivier, M. (2003). Signaling events involved in interferon-γ-inducible macrophage nitric oxide generation. *Immunology*, *108*(4), 513-522.

Chatterjee, R.N., & Rajkumar, U. (2015). An overview of poultry production in India. *Indian Journal of Animal Health*, *54*(2), 89-108.

CLC BIO. (2011). CLC Genomic Work bench 6.8.1 Aarhus, Denmark (http://www.clcbio.com).

Hadrawi, J., Gunawan, A., Ulupi, N., Darwati, S., & Sumantri, C. (2016). Association analysis of NRAMP1 gene related to resistance against *Salmonella pullorum* infection in Kampung Chicken. *In: Proceedings of the 1st International Conference Technology on Biosciences and Social Science* held on 17th–19th November, 2016 at Convention Hall, Andalas University, Padang, West Sumatera, Indonesia.

Harbison, A.M., & Nguyen, J.N.T. (2017). PCR: Identification of genetic polymorphisms. *Molecular Profiling: Methods and Protocols*, p. 193-203.

Harish Kumar, D.V. (2016). A comparative study on *cGH* gene polymorphism and their association with growth traits in Swarnadhara and Giriraja chicken. *M.V.Sc. thesis*, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, India.

Hu, J., Bumstead, N., Barrow, P., Sebastiani, G., Olien, L., Morgan, K., & Malo, D. (1997). Resistance to salmonellosis in the chicken is linked to NRAMP1 and TNC. *Genome Research*, *7*(7), 693-704.

Khosravinia, H., Murthy, H.N., Parasad, D.T., & Pirany, N. (2007). Optimizing factors influencing DNA extraction from fresh whole avian blood. *African Journal of Biotechnology*, *6*(4), 481-486.

Kramer, J., Malek, M., & Lamont, S.J. (2003). Association of twelve candidate gene polymorphisms and response to challenge with *Salmonella enteritidis* in poultry. *Animal Genetics*, *34*(5), 339-348.

Liu, W., Kaiser, M.G., & Lamont, S.J. (2003). Natural resistance-associated macrophage protein 1 gene polymorphisms and response to vaccine against or challenge with *Salmonella enteritidis* in young chicks. *Poultry Science*, *82*(2), 259-266.

Machado, M.A., Schuster, I., Martinez, M.L., & Campos, A.L. (2003). Genetic diversity of four cattle breeds using microsatellite markers. *Revista Brasileira de Zootecnia*, *32*(1), 93-98.

Malek, M., & Lamont, S.J. (2003). Association of INOS, TRAIL, TGF-2, TGF-3, and IgL genes with response to *Salmonella enteritidis* in poultry. *Genetics Selection Evolution*, *35*(Suppl. 1), S99-S111.

Muhammad, M., Niken, U., Asep, G., Teguh, W.I.W., & Cece, S. (2018). Influence of iNOS to *Salmonella Pullorum* disease resistance in Sentul Chicken. *Veterinaria*, *67*(1), 17-21.

Muhsinin, M., Ulupi, N., Gunawan, A., Wibawan, I.W.T., & Sumantri, C. (2016). Association of NRAMP1 polymorphisms with immune traits in Indonesian native chickens. *International Journal of Poultry Science*, *15*, 401-406.

Ramesh, S.N., Ramakrishnappa, N., Siddaraju, N.K., Maheshwarappa, Y.H., & Sringeshwaraiah, N.C. (2022). Genetic variations in Toll-like receptor-4 and Myeloid differentiation protein-2 genes among Giriraja and Indigenous chicken. *Indian Journal of Veterinary Sciences & Biotechnology*, *18*(2), 26-31.

Ryan, K.J., & Ray, C.G. (2014). *Sherris Medical Microbiology*. 6th edition, McGraw Hill, New York, USA.

Rychlik, I., Elsheimer-Matulova, M., & Kyrova, K. (2014). Gene expression in the chicken caecum in response to infections with non-typhoid Salmonella. *Veterinary Research*, *45*(1), 1-14.

Sadeyen, J.R., Trotereau, J., Velge, P., Marly, J., Beaumont, C., Barrow, P.A., Bumstead, N., & Lalmanach, A.C. (2004). Salmonella carrier state in chicken: Comparison of expression of immune response genes between susceptible and resistant animals. *Microbes and Infection*, *6*(14), 1278-1286.

Tohidi, R., Idris, I., Panandam, J.M., & Hair-Bejo, M. (2011). Analysis of genetic variation of inducible nitric oxide synthase and natural resistance-associated macrophage protein 1 loci in Malaysian native chickens. *African Journal of Biotechnology*, *10*(8), 1285-1289.

Tohidi, R., Idris, I.B., Panandam, J.M., & Bejo, M.H. (2012). The effects of polymorphisms in IL-2, IFN-γ, TGF-β2, IgL, TLR-4, MD-2, and iNOS genes on resistance to *Salmonella enteritidis* in indigenous chickens. *Avian Pathology*, *41*(6), 605-612.

Tohidi, R., Idris, I.B., Panandam, J.M., & Bejo, M.H. (2013). The effects of polymorphisms in 7 candidate genes on resistance to *Salmonella enteritidis* in native chickens. *Poultry Science*, *92*(4), 900-909.