

Unraveling the Genetic Diversity and Population Structure of Poonchi Chicken in the Indo-Pak Border Region through Microsatellite Markers

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ABSTRACT

The present study aimed for molecular characterization of Poonchi chicken from Poonch and Rajouri districts of Jammu and Kashmir (UT), India located near the international Border with Pakistan. Fifty blood samples were selected randomly from unrelated indigenous Poonchi chickens and analyzed for molecular characterization using ten FAO recommended microsatellite markers ADL0268, MCW0206, MCW0081, ADL0278, MCE0069, MCW0248, MCW0111, MCW0222, MCW0016 and LEI0094. The polymerase chain reaction (PCR) was standardized at annealing temperature ranging from 52°C to 60°C for different microsatellite marker. Genotyping of microsatellite markers was done at 10% denaturing urea PAGE (polyacrylamide gels electrophoresis) followed by silver staining and visualisation under UV transilluminator. Alleles obtained were analyzed to estimate genetic diversity and relationships among the populations using POPGENE 32 software. Result indicated a total of 72 alleles, with the highest 9 numbers of alleles contributed by loci MCW0069 and MCW0016, and the lowest 6 alleles contributed by ADL0268, ADL0278, and MCW0022. The average expected heterozygosity was 0.8110 ± 0.0471 . The Shannon's information index (I) ranged from 1.3766 (LEI0094) to 1.9649 (MCW0111) with the mean value 1.7648 ± 0.1915 . The average polymorphism information content (PIC) was estimated as 0.7932 ± 0.0255 . The F_{IS} values ranged from -0.2927 (LEI0094) to 0.2600 (MCW0248). The significant Hardy-Weinberg Disequilibrium (WHD) in the present study suggests that systemic and dispersive forces particularly migration and small flock size might be operating in the Poonchi chicken population. The microsatellite markers used under study captured genetic variation reliably because it was highly informative, which can be used for future genetic improvement program.

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Authors' Contribution

MSA did the planning, lab experiment, writing. DC planned the research and designed the research plan. KK was involved in sample collection and writing. NK was involved in analysis.

Key words

Hardy-Weinberg disequilibrium, PIC, POPGENE 32, Urea Page, Shannon's information index

INTRODUCTION

Increased demand for food grains and agriculture has put direct pressure on animal genetic resources of the

nation. For sustainable and efficient use, it is very essential to understand and document the diversity, population distribution, characteristics and current population status of AnGR of each country (FAO, 2007). Genetic diversity among livestock species is imperative to estimate phenotypic variation and to understand the historical development of livestock (Ajmone-Marsan *et al.*, 2010; Groeneveld *et al.*, 2010). Local native chicken populations are suited well for extreme weather conditions and have better disease resistance than exotic breeds. Earlier studies have shown that local chicken breeds exhibit better immune response than exotic breeds due to better antibody response and improved complement activity (Haunshi and Sharma, 2002; Baelmans *et al.*, 2005). Local and native

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breeds show more genetic diversity than other breeds (Plotsky *et al.*, 1995; Ponsuksili *et al.*, 1996). Proper and appropriate strategies for management of animal genetic resources are prerequisite for successful conservation and breed improvement programmes. Microsatellite markers are one of the most effective and precise method of characterization for assessing diversity and relationship among populations. Microsatellite markers are highly polymorphic, random, repetitive nucleotide units (Tautz, 1989).

Poonch is a hilly district of Jammu, India located at southern slopes of Pir Panjal and shares international borders with Pakistan. Border districts located near international borders are characterized by restricted animal movements, fragmented breeding tracts, military fencing which limits gene flow/exchange across the border. The indigenous chicken population of Poonch region is popularly known as Poonchi chicken and is located in Poonch and Rajouri districts of Jammu. Poonchi chicken (Fig. 1) is maintained under small, scattered backyard poultry system with frequent informal exchange of birds across villages with in Indian side. Poonchi poultry population is very popular among local farmers due to its hardy nature and is best suited for the tough climatic conditions as well as survives well against predation. Poonchi chicken is preferred over exotic breeds due to better taste, egg colour, flavour and meat leanness. Despite being a popular native breed among the locals and tribals no efforts were made on molecular characterization of this indigenous breed. Hence this study provides the first effort of molecular characterization using FAO recommended microsatellite markers, addressing a critical knowledge gap needed for conservation and improvement.



Fig. 1. Adult Poonchi Chicken

MATERIALS AND METHODS

A total of seventy Poonchi chickens were selected randomly from natural breeding tracts i.e different remote villages namely Gursai, Ari, Harni, Digvaar in Poonch district and Androoth, Hanjana, Guni, Rajpur Bhatta in Rajouri district of Jammu and Kashmir, India. Aseptic blood collection was done from wing vein and samples were brought to laboratory for further storage (-4°C) and analysis. Genomic DNA was isolated from the freshly collected sample using both the phenol-chloroform isoamyl (PCI) method and the Sigma Aldrich Genelute blood genomic DNA kit. 50 best DNA samples (with values ranging from 1.7 to 1.9) from the total 70 blood samples collected were selected for PCR amplification. FAO recommended microsatellite markers namely ADL0268, MCW0206, MCW0081, ADL0278, MCE0069, MCW0248, MCW0111, MCW0222, MCW0016 and LEI0094 were selected for further analysis (Table 1). The selected microsatellite markers are FAO recommended markers for genetic diversity studies in chicken population due to their high polymorphism, genome wide distribution, codominant inheritance and reproducibility across diverse indigenous and commercial poultry breeds. PCR conditions were optimized for all 10 microsatellite markers and PCR amplification was carried out for all 50 samples using each marker separately. The final volume of 25 μl was prepared with genomic DNA (2.5 μl), forward primer (1.5 μl), reverse primer (1.5 μl), 2X PCR master mix (12.5 μl), nuclease free water (7 μl). The amplified products were checked on a 3.0% agarose gel with a 50 and 100 bp ladder as a marker. The positive samples with specific amplification were further resolved on 10% urea polyacrylamide gel electrophoresis for a period of 4 hours at 100-120 volts. The gels were then stained with silver staining and visualized under UV transilluminator. Alleles and probable genotypes were recorded in ascending order of size and numbered alphabetically as A, B, C... etc. Each allele was compared with 100 bp or 50 bp DNA ladder co-migrated in the same gel. The number of alleles in each sample were analysed using statistical software Popgen32, for estimating genetic diversity and relationships among the populations. Data was obtained for genotype frequency, allele frequency, deviation from Hardy-Weinberg equilibrium (HWE), observed number of alleles (n_a), effective number of alleles (n_e), Shannon's informative index (I), observed heterozygosity (H_o), expected heterozygosity (H_e), Polymorphic information content (PIC), Wright's fixation indices (F_{is}), Ewens-Watterson Test for neutrality and Smouse's multilocus analysis for single population.

Table I. Primer sequences of different microsatellite used in the study.

S. No.	Chromo some	Markers	Primer sequence (5'-3')	Primer size	Product size (bp)
1	1	<i>ADL0268</i>	CTCCACCCCTCTCAGAACTA CAACTTCCCCTACCTACT	20	102-116
2	2	<i>MCW0206</i>	CTTGACAGTGATGCATTAAATG ACATCTAGAATTGACTGTTTAC	22	221-249
3	5	<i>MCW0081</i>	GTTGCTGAGAGCCTGGTGCAG CCTGTATGTGGAATTACTTCTC	21	112-135
4	8	<i>ADL0278</i>	CCAGCAGTCTACCTTCCTAT TGTCATCCAAGAACAGTGTG	20	114-126
5	26	<i>MCW0069</i>	GCACTCGAGAAAACTTCCTGCG ATTGCTTCAGCAAGCATGGGAGGA	22	158-176
6	1	<i>MCW0248</i>	GTTGTTCAAAAGAAGATGCATG TTGCATTAACCTGGCACTTTC	22	205-225
7	1	<i>MCW0111</i>	GCTCCATGTGAAGTGGTTTA ATGTCCACTTGTCATGATG	20	96-120
8	3	<i>MCW0222</i>	GCAGTTACATTGAAATGATTCC TTCTCAAAACACCTAGAAGAC	22	220-226
9	3	<i>MCW0016</i>	ATGGCGCAGAAGGCAAAGCGATAT GGCTTCTGAAGCAGTTGCTATGG	24	162-206
10	4	<i>LEI0094</i>	GATCTCACCAGTATGAGCTGC TCTCACACTGTAACACAGTGC	21	247-287

Table II. Significance of different microsatellite markers for HWE.

Locus/ Markers	χ^2 Value	χ^2 Significance	G-square value	G-square significance
ADL0268	55.639847	0.000001**	66.549832	0.000000**
MCW0206	55.374702	0.000062**	63.123894	0.000004**
MCW0081	66.219914	0.000062**	64.944801	0.000092**
ADL0278	47.566099	0.000030**	48.399016	0.000022**
MCW0069	55.628416	0.019409*	61.249776	0.005412**
MCW0248	62.943481	0.000005**	56.965167	0.000036**
MCW0111	80.863094	0.000000**	77.646103	0.000001**
MCW0222	82.089294	0.000000**	84.170762	0.000000**
MCW0016	93.202510	0.000001**	90.037537	0.000002**
LEI0094	30.711853	0.009598**	28.570067	0.018261*

**, P<0.01; *, P<0.05

RESULTS

The present study aims to genetically characterize the indigenous Poonchi chicken population reared in natural breeding tract of Poonch and Rajouri districts of Jammu and Kashmir, India representing a unique population

located near to Indo-Pak border borders. This is the first genetic diversity study done on Poonchi Chicken using FAO recommended microsatellite markers.

Since early 1990s microsatellite markers are the most commonly used genetic markers for diversity studies (Ashley and Dow, 1994; Bruford and Wayne, 1993; Li

et al., 2002). Poonchi chicken population is one of the first poultry population being characterized from Jammu province of Jammu and Kashmir, India. However, like many other indigenous breeds, Poonchi chicken is facing threats from the widespread use of commercial breeds in poultry farming. Since very scanty information regarding this indigenous population is available, it is very important to characterize and promote this breed as well as to educate farmers on the importance of conserving this breed. Therefore, the present study was carried out for molecular characterizations of Poonchi chicken using ten FAO recommended microsatellite markers. Popgen32 software was used to calculate allele frequency, observed number of alleles (n_a), effective number of alleles (n_e), Shannon's informative index (I), observed heterozygosity (H_o), expected heterozygosity (H_e), PIC and Wright's fixation indices (F_{is}). Test for Hardy-Weinberg Equilibrium for ADL0268, MCW0206, MCW0081, ADL0278, MCW0069, MCW0248, MCW0111, MCW0222, MCW0016, and LEI0094 microsatellite markers have been presented in Table II. The results indicated that chi-square and G-square values were highly significant ($P < 0.01$) for all the microsatellite markers under present study except for MCW0069, where the χ^2 value was only significant ($P < 0.05$) and for LEI0094 G-square value was only significant ($P < 0.05$).

Table III. Genetic variation statistics of all markers in the indigenous Poonchi chicken population.

Locus	n_a *	n_e *	I *	PIC
ADL0268	6.0000	4.1632	1.5656	0.7257
MCW0206	7.0000	5.5866	1.8033	0.7963
MCW0081	8.0000	6.6138	1.9568	0.8304
ADL0278	6.0000	4.8685	1.6742	0.7644
MCW0069	9.0000	6.0168	1.9112	0.8126
MCW0248	7.0000	4.7664	1.7362	0.7645
MCW0111	8.0000	6.6756	1.9649	0.8323
MCW0222	6.0000	5.3648	1.7243	0.7866
MCW0016	9.0000	5.5804	1.9353	0.9751
LEI0094	6.0000	3.2916	1.3766	0.6348
Mean	7.2000	5.2928	1.7648	0.7932
St. Dev	1.2293	0.9235	0.1915	0.0255

n_a , Observed number of alleles; * n_e , Effective number of alleles Kimura and Crow (1964); * I = Shannon's Information index Lewontin (1972).

Genetic variation statistics for all loci of the present study in Poonchi chicken have been presented in Table II.

The PIC Polymorphism information content) values have been presented in Table III. A graphical representation of variation in allele frequency for all 10 markers is depicted. High allele frequency was due to the large number of alleles because of increased heterozygosity. All 10 markers used were highly informative and are very useful for molecular characterization or genetic diversity studies.

The summary of heterozygosity statistics of all markers in the Poonchi chicken is presented in Table III. The Nei's values are presented in Table IV. Wright fixation index was calculated to measure population variation and inbreeding coefficient. Out of 72 alleles observed, 53 showed negative F_{is} value (Fig. 2). Average F_{is} or inbreeding coefficient was -0.0416 which indicates absence of inbreeding coefficient and presence of high heterozygosity. The reason of increase heterozygosity is outbreeding and migration because of introduction of breeding male and female from outside into the population.

Table IV. Heterozygosity statistics of all markers in the local Poonchi chicken population.

Locus	Obs_Hom	Obs_Het	Exp_Hom*	Exp_Het*	Nei**	Ave_Het
ADL0268	0.0400	0.9600	0.2325	0.7675	0.7598	0.7598
MCW0206	0.0200	0.9800	0.1707	0.8293	0.8210	0.8210
MCW0081	0.2800	0.7200	0.1426	0.8574	0.8488	0.8488
ADL0278	0.2400	0.7600	0.1974	0.8026	0.7946	0.7946
MCW0069	0.2000	0.8000	0.1578	0.8422	0.8338	0.8338
MCW0248	0.4200	0.5800	0.2018	0.7982	0.7902	0.7902
MCW0111	0.0600	0.9400	0.1412	0.8588	0.8502	0.8502
MCW0222	0.1000	0.9000	0.1782	0.8218	0.8136	0.8136
MCW0016	0.0200	0.9800	0.1709	0.8291	0.8208	0.8208
LEI0094	0.1000	0.9000	0.2968	0.7032	0.6962	0.6962
Mean	0.1480	0.8520	0.1890	0.8110	0.8029	0.8029
St. Dev	0.1331	0.1331	0.0471	0.0471	0.0466	0.0466

*Expected homozygosity and heterozygosity were computed using Levene (1949). ** Nei's (1973) expected heterozygosity.

The Ewens-Watterson test is very important test and was one of the first tests of the neutral theory i.e., of no selection. The Ewens-Watterson test for neutrality was performed in 1000 simulated data by using PopGene 32 software. The results depicted in Table V, indicated that the results for ADL0268, MCW0206, MCW0081, ADL0278, MCW0069, MCW0248, MCW0111, MCW0222, MCW0016, and LEI0094 were non-significant for Ewens-Watterson test for neutrality.

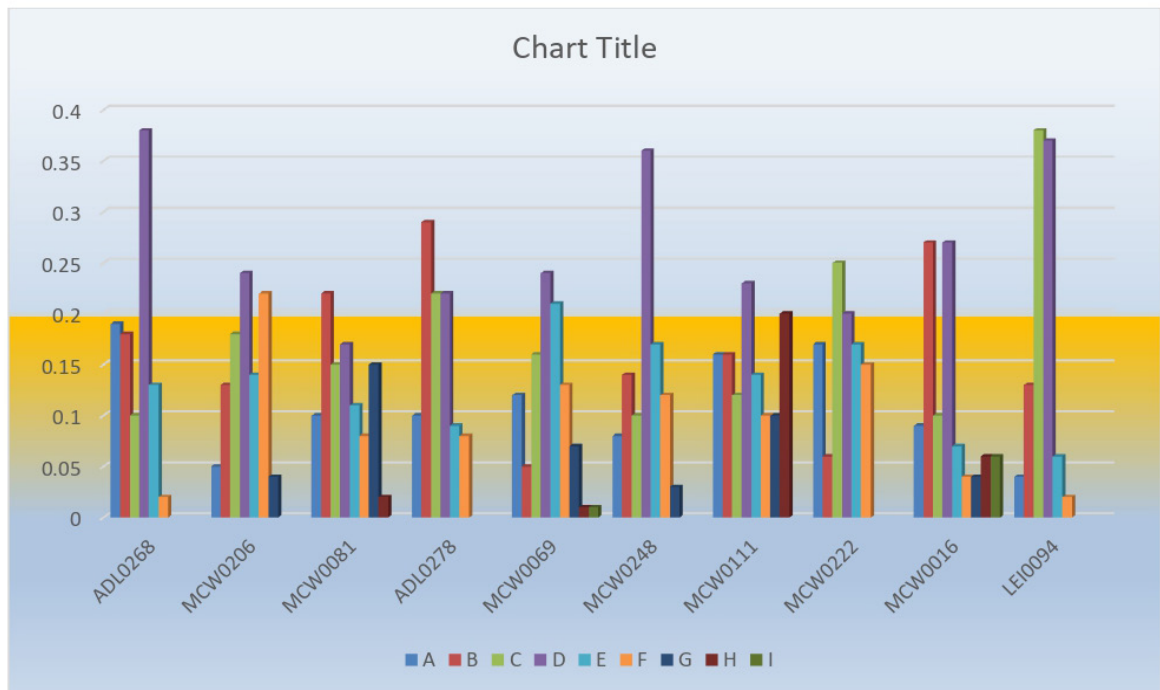
Table V. Ewens-Watterson test for neutrality of microsatellite loci in the local Poonchi chicken population.

Locus	k	Obs. F	Min F	Max F	Mean*	SE*	L95*	U95
ADL0268	6	0.2402	0.1667	0.9050	0.4226	0.0198	0.2358	0.7460
MCW0206	7	0.1790	0.1429	0.8872	0.3726	0.0162	0.2068	0.7018
MCW0081	8	0.1512	0.1250	0.8698	0.3329	0.0129	0.1850	0.6338
ADL0278	6	0.2054	0.1667	0.9050	0.4221	0.0193	0.2266	0.7460
MCW0069	9	0.1662	0.1111	0.8528	0.3004	0.0117	0.1670	0.5878
MCW0248	7	0.2098	0.1429	0.8872	0.3782	0.0190	0.2084	0.7438
MCW0111	8	0.1498	0.1250	0.8698	0.3289	0.0131	0.1822	0.6536
MCW0222	6	0.1864	0.1667	0.9050	0.4262	0.0205	0.2300	0.7614
MCW0016	9	0.1792	0.1111	0.8528	0.2991	0.0102	0.1738	0.5592
LEI0094	6	0.3038	0.1667	0.9050	0.4214	0.0208	0.2270	0.7622

F- F-values; L95-Lower confidence limits at 95% confidence; U95-Upper confidence limits at 95% confidence.

Table VI. Smouse's multilocus analysis for single population.

Population	Individuals	Ave. Corr.	df	Chi-square	Prob.	WHD*	df	Chi-square	Probability
1	50	0.2143	45	54.92	0.1476	-0.0945	10	35.14	0.0001

**Fig. 2. Wrights (1978) fixation index (F_{IS}) for microsatellite loci in the local Poonchi chicken.**

Smouse's multilocus analysis showed non-significant chi-square values for average correlation and highly significant WHD values. The analysis results can be seen in [Table VI](#).

DISCUSSION

Poonchi chicken is very famous in Jammu region as native Poonch chicken but till now very less work has

been done on this native population due to which there is almost very less to no information available about this important breed. This is the first native poultry breed of the region to be characterized and registered with national breed registration organization. This study was mainly carried out with the aim of characterization of this unique breed. Similar studies have been carried out by different researchers across the globe using microsatellite markers for characterization of various native breeds. Similar to the present study, in one or more number of microsatellite markers higher number of alleles were observed by [Fernandes *et al.* \(2009\)](#) in Eastern and Northern Red Jungle Fowl, [Mukesh *et al.* \(2011\)](#) in Indian Jungle fowl [Hillel *et al.* \(2003\)](#) in chicken breeds worldwide, [Lujang *et al.* \(2006\)](#) in various chicken breeds of China, [Granevitze *et al.* \(2007\)](#) in Chinese jungle fowl, [Bao *et al.* \(2008\)](#) in Chinese jungle fowl, [Mukesh *et al.* \(2011\)](#) in Indian jungle fowl, [Dorji *et al.* \(2012\)](#) in chicken breeds of Bhutan, [Azimu *et al.* \(2018\)](#) in local chicken of China and [Habimana *et al.* \(2020\)](#) in chicken breeds of Rwanda. The consistent observation of lower effective number of alleles (n_e) compared to observed number of alleles (n_a) across all loci suggest frequency skewness, where few alleles occur at high frequency while other remain rare. This pattern is typical of indigenous backyard poultry populations. As Poonchi chicken is reared in small flocks and there is no systematic breeding knowledge among farmers, inbreeding occurs in the population causing reduction of genetic diversity over time and increasing the likelihood of genetic bottlenecks.

The Poonchi bird is unique in nature and after independence some part of natural habitat goes to Pakistan. That left with a small group of individuals which subsequently establishes a new population; hence the genetic variation is limited to Poonchi chicken leading to a genetic bottleneck. Despite this skewness, the high heterozygosity and PIC value indicate that overall genetic diversity remains well preserved.

All the microsatellite loci under present study showed highly significant ($P < 0.01$) chi-square and G-square values for all the microsatellite markers except for MCW0069, where the χ^2 value was only significant ($P < 0.05$). Similarly to the present study significant effect was reported by [Abebe *et al.* \(2015\)](#) for ADL0268, MCW0081, MCW0069, MCW0248 in five Sweden chicken population, [Soltan *et al.* \(2016\)](#) for ADL0268, ADL0278, MCW0248 in chicken population of Egypt, [Azimu *et al.* \(2018\)](#) for ADL0268, ADL0278, MCW0206, MCW0069, MCW0111, MCW0222, MCW0016, LEI0094, MCW0248 in eight different chicken populations of China, [Habimana *et al.* \(2020\)](#) for ADL0268, MCW0016 in chicken population of Rwanda, [Ding *et al.* \(2010\)](#) for ADL0278, MCW0278,

MCW0248 in Bian, Jinghai and Youxi chicken population of China, [Rahim *et al.* \(2017\)](#) for MCW0069 in Rhode Island Red chicken, [Palacios *et al.* \(2016\)](#) for ADL0278, MCW0081, MCW0248 in Creole birds of Columbia, [Possamai *et al.* \(2015\)](#) in Paraiso Pedres for MCW0081, LEI0094 and [Rashid *et al.* \(2020\)](#) for LEI0094 in chicken of Bangladesh. The significant values suggest that the population was not in Hardy-Weinberg equilibrium. This indicates that systematic as well as dispersive forces may be operating in the population and it can be concluded that population may undergo bottleneck effect.

All the Shannon index values for all 10 markers were very high and above 1.5 except for LEI0094 indicating highly polymorphic nature of all the markers. The overall mean values were 1.7648 with standard deviation of 0.1915. This shows all 10 microsatellite markers were highly informative. Similar to this higher Shannon's information index (I) values were reported by [Pirany *et al.* \(2007\)](#) MCW0016 in Indian chicken population, [Mukesh *et al.* \(2011\)](#) for ADL0268 in Red Jungle fowl of India and [Habimana *et al.* \(2020\)](#) ADL0268, ADL0278 and LEI0094 in chicken population of Rwanda. In contrast to this lower Shannon's information index (I) values were reported by [Keambou *et al.* \(2014\)](#) for ADL0268, MCW0206, MCW0081, ADL0278, MCW0069, MCW0248, MCW0222 and LEI0094 in chicken population of Cameroon, [Mukesh *et al.* \(2011\)](#) for MCW0111, MCW0222 and MCW0081 in Red Jungle fowl of India, [Pirany *et al.* \(2007\)](#) ADL0278 and MCW0069 in Indian chicken population, [Rahim *et al.* \(2017\)](#) for MCW0069 in Rhode Island Red chicken, [Das \(2012\)](#) for MCW0016 in RIR^s and RIR^c populations, [Pandey *et al.* \(2005\)](#) for MCW0016 in Ankleshwar chicken and [Habimana *et al.* \(2020\)](#) for MCW0206, MCW0081, MCW0069, MCW0248, MCW0111, MCW0222 and MCW0016 in chicken population of Rwanda.

The overall PIC observed were higher than 0.5 which clearly show that all the 10 markers under study were highly polymorphic in nature. The results of the present study indicate the genetic efficiency of these markers in indigenous chicken population genetic diversity studies. Similar higher PIC values were reported by [Hillel *et al.* \(2003\)](#), [Lujang *et al.* \(2006\)](#), [Nasiri *et al.* \(2007\)](#), [Bao *et al.* \(2008\)](#), [Kaya and Yildiz \(2008\)](#), [Mukesh *et al.* \(2011\)](#), [Keambou *et al.* \(2014\)](#), [Abebe *et al.* \(2015\)](#), [Choi *et al.* \(2015\)](#), [Azimu *et al.* \(2018\)](#), [Ozdemir and Cassandro \(2018\)](#), [Choi *et al.* \(2019\)](#), [Habimana *et al.* \(2020\)](#), [Rashid *et al.* \(2020\)](#), [Pham *et al.* \(2013\)](#), [Seo *et al.* \(2017\)](#).

The results from this study clearly indicates that Poonchi chicken population possesses sufficient genetic diversity for long term sustainability. The observed high heterozygosity, negative mean Fis values and deviation from Hardy-Weinberg equilibrium in Poonchi chicken

indicate outbreeding and absence of systematic selection. High average PIC values, high Shannon information index collectively indicate a genetically diverse, non-inbred population capable of adaptation to environmental and disease challenges in future. It is very important to conserve this native breed through a planned region-specific breeding plan which involves following points: (i) Awareness among the farmers- farmers should be trained for scientific breeding management among Poonchi chicken so that inbreeding can reduce in the population. (ii) Introduction/exchange of breeding cock and hen- breeding individuals from genetically diverse Poonchi chicken population should be introduced or farmers should exchange the breeding birds among themselves to reduce the inbreeding and enhance genetic variation. (iii) Phenotype assisted selection focusing on survivability, disease tolerance and production. (iv) In situ conservation under backyard production system supported by government agencies and institutions.

DECLARATIONS

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Ethical statement

The study followed all local and national ethical standards. It was carried out in a non-invasive manner to minimize disturbance to the birds and their natural habitat.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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