Original Article

MHC-linked microsatellite LEI0258 variability and population structure of chicken ecotypes in Iran

Ramezani, A.[1]; **Nikbakht Brujeni, G.**[2*]; **Sheikhi, N.**[3]
and Parvandar Asadollahi, K.[3]

[1]Ph.D. Student in Immunology, Department of Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, Iran;
[2]Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [3]Department of
Clinical Science, Science and Research Branch, Islamic Azad University, Tehran, Iran

*Correspondence: G. Nikbakht Brujeni, Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of
Tehran, Tehran, Iran. E-mail: nikbakht@ut.ac.ir

Abstract

Background: Investigation of chicken major histocompatibility complex (MHC) genes along with their diversity across native regions and populations, as a genetic resource, can be used in the breeding programs. Important characteristics of MHC genes, such as the association with immunological and production traits, make them exceptional candidates for marker assisted selection. **Aims:** The aim of this study was to characterize MHC based on the LEI0258 microsatellite marker to evaluate the genetic variability and diversity within and between Iranian chicken populations. **Methods:** Blood samples were collected from six indigenous ecotypes (n=633) and Ross 308 (n=216) as a commercial breed. The MHC variability was determined based on a microsatellite marker located within MHC, LEI0258. Polymerase chain reaction and fragment analysis was used for microsatellite polymorphism detection. **Results:** Based on the fragment analysis, 7 alleles were found in Ross 308 and 25 alleles across all 6 populations. The population with the maximum genetic diversity was Mazandaran (0.939), while the population with the minimum genetic diversity was Ross 308 (0.794). Out of the 6 Iranian chicken ecotypes, all except Arian and Khorasan, were in Hardy-Weinberg equilibrium (P<0.05). The genetic variations within (84.92%) and between (15.08%) populations were statistically significant (P<0.001). **Conclusion:** A significant genetic structure that is not completely homogeneous among the Iranian chicken populations can be considered as distinct genetic resources. This study highlights the value of using markers such as LEI0258 to investigate the diversity of genes that play dual roles in immunity and production.

Key words: Chicken, Major histocompatibility complex, LEI0258, Iran

Introduction

The major histocompatibility complex (MHC) of chicken, assigned as B complex, originally described as the second highly polymorphic blood group system. MHC was later found to determine not only the erythroid alloantigens, but also the acute allograft rejection and genetic control of the innate and adaptive immune responses (Fulton *et al*., 2006). The chicken MHC genes are split into two regions of micro chromosome 16; classical *MHC* genes are located in the B complex region and Rfp-Y region composed of non-classical genes. The B complex is approximately 92 kb with only 19 genes among which BF (class I), BL (class IIβ) and BG (class IV) are of the tightly linked and highly polymorphic regions (Yuan *et al*., 2021).

Several important characteristics of *MHC* genes make them exceptional candidates for investigation. *MHC* genes play an important role in immune system,

autoimmunity, economic traits, reproductive success and life history strategies. Identification of chicken MHC haplotypes and diversity across native regions and populations, as a genetic resource of each country, would be worthwhile and can be used in the future breeding programs (Chang *et al*., 2012; Wang *et al*., 2014; Kannaki *et al*., 2017). To simultaneously improve immunological and production traits, molecular markers associated with one or both set of traits are of great importance. In this situation, chicken B complex as a marker assisted selection (MAS) is expected to be a more effective breeding approach.

Hemagglutination (HA) with specific complex B alloantisera was the foremost method for identifying B haplotypes. Although HA typing could be used for the detection of expressed genes, preparing the alloantisera needs labor characterization and technical validations. Furthermore, since specific alloantisera for each B haplotype are complex mixtures of antibodies, often

exhibit extensive cross-reaction when used for different chicken populations. Several molecular methods are now available to identify B haplotypes and genotypes in chicken. DNA-based identification methods that have been well developed for specified loci are sequence specific primers (PCR-SSP), single strand conformation polymorphism (SSCP), and restriction fragment length polymorphism (RFLP). Each of these methods is useful to determine the specific B loci, but not the haplotypes or whole B complex region. Single nucleotide polymorphism (SNP) and variable number tandem repeat (VNTR) are being used for reliable and rapid means of determining haplotypes (Zheng *et al*., 1999; Fulton, 2020).

A tetra nucleotide repeat system assigned as microsatellite marker LEI0258 is a VNTR located within the chicken MHC region. Due to its close physical location and strong genetic linkage disequilibrium to genes of MHC, this marker was investigated as a genetic indicator for haplotypes (Lima-Rosa *et al*., 2005; Fulton *et al*., 2006). MHC typing using polymorphic micro-satellite marker LEI0258 was first described by Fulton *et al*. (2006). Later, several studies used this marker for MHC typing. In Iran, Izadi *et al*. (2011) investigated genetic diversity of the MHC region in commercial and noncommercial chicken flocks using the LEI0258 microsatellite marker (Izadi *et al*., 2011). They found that noncommercial populations harbored more alleles than commercial populations. The aim of this study was to characterize the B complex haplotypes based on the LEI0258 microsatellite marker to evaluate the genetic variability and diversity within and between 6 Iranian chicken ecotypes and one commercial Ross 308 population.

Materials and Methods

Sampling and DNA extraction

Blood samples of indigenous chicken ecotypes were provided by the Iranian research centers on indigenous chickens, Ministry of Agriculture. Blood samples included: 58 Arian samples from Amol in Mazandaran, 312 Khorasan samples from Central Khorasan, 43 Marandi samples from East Azarbaijan, 74 Mazandaran samples from Mazandaran, 67 Urmia samples from West Azerbaijan, and 79 Esfahan samples from Esfahan provinces. The detailed history and genetic differences of Iranian indigenous chicken have been studied and reported previously (Shahbazi *et al*., 2007). A total of 633 blood samples were collected from Iranian ecotypes and 216 samples from Ross 308 commercial chicken flocks located in Tehran province. Blood samples were taken from the ulnar vein and stored in vacuum tubes containing EDTA at -20°C before extraction. Genomic DNA was extracted from whole blood using AccuPrep Genomic DNA Extraction Kit (Bioneer, Korea), according to manufacturer's instructions.

Polymerase chain reaction

The MHC haplotype was determined based on a microsatellite marker located within MHC, LEI0258. The mixtures for PCR reactions, and reagent concentrations, were as follows: 20 ng of genomic DNA, with 0.8 µL of each primer as described by McConnell *et al*. (1999) (GenBank Z83781), 1.5 mM of MgCl$_2$ and 0.2 µL of Taq in manufacture's PCR buffer (CinaClon, Tehran, Iran) in a final volume of 25 µL. The sequences of forward and reverse primers were CAC GCA GCA GAA CTT GGT AAG G and AGC TGT GCT CAG TCC TCA GTG C, respectively.

A dye-labeled of the forward primer with 6-Fam was used to produce a PCR product labeled with fluorescence. The amplification process involved an initial denaturation at 94°C for 1 min, followed by 35 cycles of 92°C for 45 s, 57°C for 45 s, and 72°C for 45 s, concluding with a 10 min extension at 72°C.

Fragment analysis

For the fragment analysis, amplified PCR product (0.5 µL) was added to 9 µL HiDi fomamide dye and 0.5 µL GeneScan 500 LIZ® Size Standard (Applied Biosystems) and the mixture was maintained at 95°C for 3 min. Allele size scoring of fluorescently labeled amplicons were determined using an ABI 3130 XL genetic analyzer (Applied Biosystems, Foster City, CA, USA) and fragments size were read using GeneMarker software (GeneMarker v1.6, Soft Genetics LLC, State College, PA).

Population genetic analysis

The program POPGENE version 1.32 was used to calculate gene and genotypic frequencies (Yeh and Boyle, 1997). Number of observed allele (Na), effective number of alleles (Ne), expected and observed homozygosity and heterozygosity for each population were measured as described by Levene (1949) and Nei (1973). Deviations from Hardy-Weinberg (HW) equilibrium were estimated by the FIS parameter (Weir and Cockerham, 1984).

At a second step, web version of POPTREE (Takezaki *et al*., 2014) was used in order to draw appropriate phylogenetic tree diagrams based on the mean number of pairwise differences and constructing population trees from allele frequencies. This web tool implements the UPGMA method assuming that all populations exist simultaneously and follow an evolutionary clock. Consequently, the branches of the tree are not allowed to vary in length arbitrarily; instead, they are restricted so that the total length from the tree root to any population remains constant.

Results

Figure 1 illustrates the results of allele size scoring for microsatellite LEI0258 through fragment length analysis. Based on the results, Table 1 was constructed showing the frequency of certain LEI0258 alleles in Iran. The Table 1 includes Ross 308 as a commercial breed as well as Khorasan, Marandi, Arian, Mazandaran, Uremia, and Esfahan chicken ecotypes within Iran.
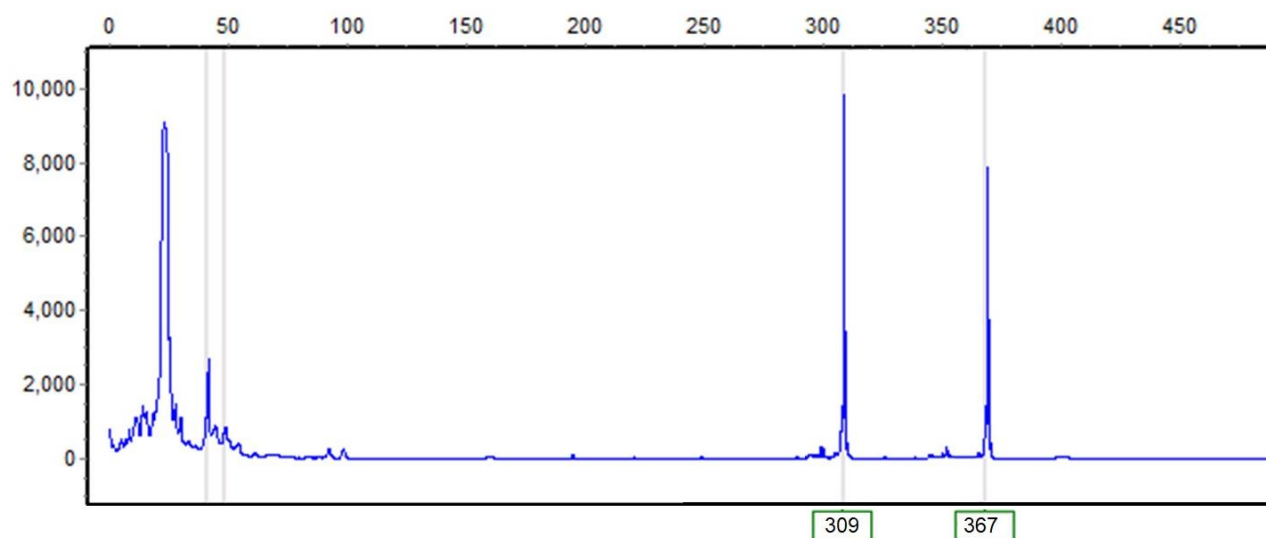
Fig. 1: Allele size scoring of microsatellite LEI0258 by fragment length analysis. PCR amplification with 6-FAM labelled primer performed on genomic DNA extracted from chicken and the size of labeled amplicons were determined using an ABI 3130 XL genetic analyzer. Fragments size were read using GeneMarker software (GeneMarker v1.6, Soft Genetics LLC, State College, PA). A representative sample indicated two bans of 309 and 367 bp

Table 1: Frequency of certain LEI0258 microsatellite alleles in chicken ecotypes of Iran. The alleles are represented by numbers, and each allele has a corresponding frequency value. The frequency values represent the percentage of individuals within each ecotype that possess a specific allele. The "Overall Freq" column provides the overall frequency of each allele across all the ecotypes listed in the table

				Ecotypes				
Allele	Ross 308	Khorasan	Marandi	Arian	Mazandaran	Urumia	Esfahan	Overall Freq.
182		0.0016				0.0224	0.0063	0.004
194		0.0207	0.0119	0.1724	0.02	0.0149	0.0633	0.039
195	0.103							
205		0.1035	0.0833	0.1466	0.007	0.0672	0.0253	0.083
206								
207	0.250							
223		0.0382	0.0357	0.0431	0.108	0.1119	0.0506	0.056
230		0.0111			0.041	0.0746	0.019	0.021
247		0.043	0.0714		0.054	0.0448	0.0316	0.041
261		0.0541	0.0833	0.2759	0.088	0.1418	0.0633	0.09
263	0.012							
273		0.0048			0.014	0.2239	0.0316	0.032
295		0.0478	0.0476	0.0345	0.061	0.0672	0.1203	0.058
300	0.052							
309		0.1067	0.0238	0.0172		0.0299	0.038	0.064
321		0.2293	0.0476		0.054		0.057	0.129
333			0.1429		0.068	0.0149	0.0316	0.023
345		0.0255	0.0476		0.047	0.0149	0.0127	0.024
357		0.0398	0.0238	0.0172	0.095	0.0224	0.038	0.041
362	0.136							
367		0.1608	0.0357	0.069	0.054	0.0448	0.0949	0.112
381		0.0127	0.0119		0.034	0.0224	0.1139	0.028
385	0.355							
393		0.0239	0.0833	0.0776	0.027		0.0443	0.033
405		0.0111	0.0238		0.041	0.0149	0.019	0.016
420		0.0143	0.0476		0.047	0.0075	0.057	0.024
443		0.0414	0.0357	0.1207	0.027	0.0075	0.0506	0.044
448	0.092							
474		0.0096	0.0476	0.0259	0.02	0.0224	0.0127	0.017
487			0.0238		0.027	0.0299	0.019	0.01
513			0.0238		0.02			0.004
539			0.0238					0.002
552			0.0238		0.047			0.007

There was a total of 7 alleles found in Ross 308 and 25 alleles across all 6 populations, with sizes ranging from 182 to 552 bp (Table 1). Among the Iranian chicken ecotypes, the number of alleles ranged from 10 (Arian) to 22 (Marandi and Esfahan) with an average of 19 alleles per ecotype. Based on the overall frequency, among the listed alleles, allele 321 is the most prevalent (0.129) with the highest occurrences found in Khorasan ecotype. Allele 539 has the lowest frequency (0.002) across the different ecotypes in Iran. Nine alleles (194, 205, 223, 261, 295, 357, 443, and 474 bp) were common to all six local chicken populations (Table 1).

According to the results of analysis, Mazandaran chickens had the highest effective number of alleles (16.619), while Arian chickens had the lowest (6.371). The overall means of observed (Obs_Hom) and expected homozygosity (Exp_Hom) were 0.229 and 0.107, respectively. The overall observed (Obs_Het) and expected (Exp_Het) heterozygosity were 0.772 and 0.895, respectively. The Arian population exhibited the highest level of Obs_Hom (65.5%), whereas the Ross 308 showed the lowest Obs_Hom (3.1%) among the populations studied. The population with the highest Obs_Het was the Ross 308 (97%), and the lowest was Arian (34.5%). The polymorphism information content (PIC) at LEI0258 microsatellite marker was highest for Esfahan (0.946) and lowest for Khorasan ecotypes (0.107). Uremia was classified as having good diversity (0.901), while Arian, Marandi, and Mazandaran ecotypes displayed high genetic diversity, with values of 0.772, 0.895, and 0.895, respectively. Ross 308, as a commercial breed, exhibited moderate genetic diversity (PIC: 0.229). The population with the maximum genetic diversity according to the Nei index was Mazandaran (0.939), whereas the population with the minimum genetic diversity was Ross 308 (0.794). Out of the 7 Iranian chicken ecotypes, all except Arian and Khorasan

were in Hardy-Weinberg equilibrium (HWE) (P<0.05) (Table 2). Results from the analyzing molecular variance (AMOVA) indicated that the genetic variation among the indigenous populations was 15.08%. It is slightly higher as compared with the analysis of all populations including Ross 308 (13.34%). The genetic variation within the indigenous populations was 84.92% that is slightly lower than the analysis of all populations including Ross 308 (86.66%). Both values were statistically significant (P<0.001), indicating that the genetic structure among the populations is still significant even without the Ross 308 population.

The tree was constructed using genetic distances matrix in Table 3. The phylogenetic tree built from the LEI0258 alleles found in Iran chicken ecotypes discriminated three main clusters with two sub-clusters (Fig. 2). The clustering of LEI0258 marker alleles based on the allelic frequency indicated that Arian, Urmia, Khorasan and Esfahan located at one cluster with two sub-clusters. Marandi and Mazandaran seem to cluster together and Ross 308 as a unique separate cluster.

In the second step, we gathered available data from Iran and three world chicken populations including Tanzania, Nigeria and Indonesia (Mwambene et al., 2019; Olufowobi et al., 2020; Mustofa et al., 2022). The phylogenic tree was constructed using the allele frequency. The clustering of LEI0258 marker alleles based on chicken geographic origin was shown in Fig. 3. The tree built from the LEI0258 alleles found in Iran, Tanzania, Nigeria and Indonesia discriminated three main clusters: one private cluster with two sub-clusters of 3 Indonesian chicken ecotypes (Sentul, Gaga, and Nunukan); one cluster with two sub-clusters including chicken ecotypes of Nigeria (Dourgou, Goggori, Tchagara, Kolonto, and Popular) and Indonesia (Merawang and Pelung); and one cluster containing all Iranian and Tanzanian ecotypes along with one

Table 2: Analysis of genetic diversity in seven Iranian chicken populations

Population	N	Na	Ne	Obs_Hom	Obs_Het	Exp_Hom	Exp_Het	PIC	Nei	HWE
Ross 308	216	7	4.845	0.031	0.971	0.211	0.799	0.229	0.794	*
Arian	58	11	6.371	0.655	0.345	0.149	0.851	0.772	0.843	*
Khorasan	312	20	8.715	0.223	0.777	0.113	0.887	0.107	0.885	*
Marandi	43	22	15.207	0.191	0.811	0.055	0.946	0.895	0.934	NS
Mazandaran	74	22	16.619	0.162	0.838	0.054	0.946	0.895	0.939	NS
Uremia	67	20	9.421	0.134	0.866	0.099	0.901	0.901	0.894	NS
Esfahan	79	21	12.779	0.205	0.796	0.068	0.932	0.946	0.922	NS
Total	849	123								
Mean	121.286	17.571	10.565	0.229	0.772	0.107	0.895	0.753	0.887	

N: Number of samples, Na: Number of alleles, Ne: Effective population size, Obs_Hom: Observed homozygosity, Obs_Het: Heterozygosity, Exp_Hom: Expected homozygosity, Exp_Het: Expected heterozygosity, PIC: Polymorphism information content, Nei: Nei's (1973) expected heterozygosity, HWE: Test for Hardy-Weinberg equilibrium, P<0.05, * Significant, and NS: Non-significant

Table 3: Genetic distances of Iranian chicken populations obtained from allele frequency data

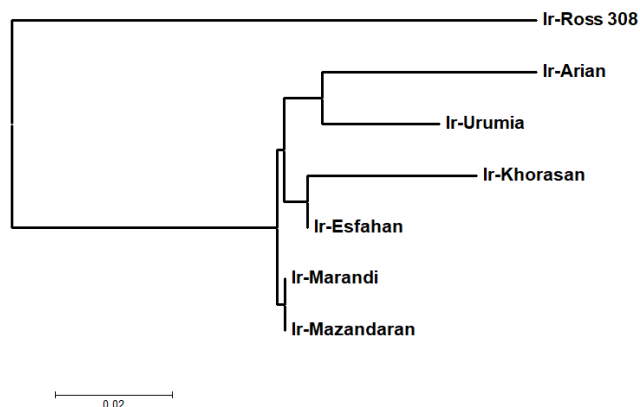| Ecotypes	Khorasan	Marandi	Arian	Mazandaran	Uremia	Esfahan
Ross 308	0.169	0.134	0.184	0.136	0.159	0.140
Khorasan		0.033	0.074	0.036	0.066	0.028
Marandi			0.039	-0.002	0.033	0.007
Arian				0.050	0.057	0.044

Fig. 2: Neighbor-Joining tree of the alleles defined for LEI0258 of chicken ecotypes in Iran. The tree was generated using allelic frequency data
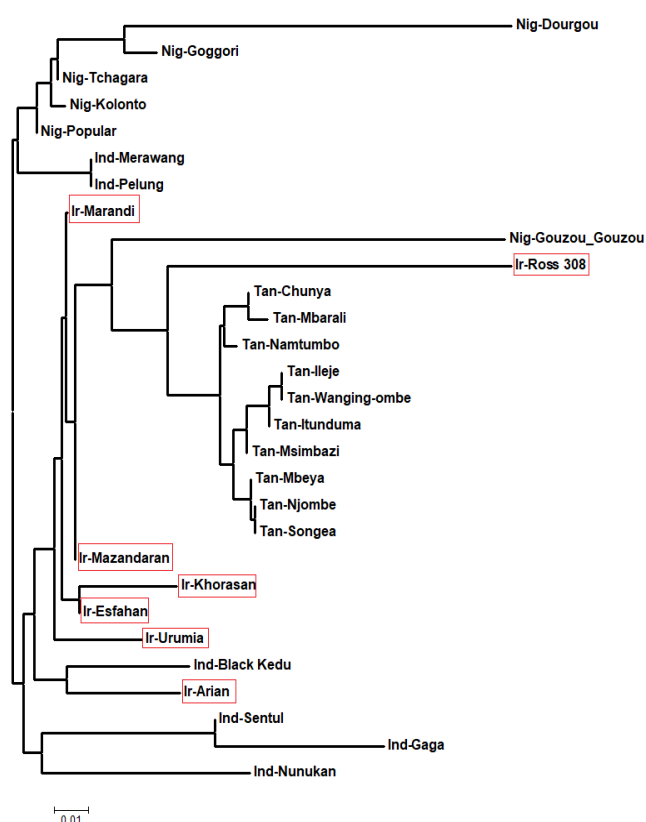

Fig. 3: Neighbor-Joining tree of the alleles reported for LEI0258 of the chicken ecotypes in Iran, Tanzania, Nigeria, and Indonesia

Indonesian (Black Kedu) and one Nigerian (Gouzo-Gouzo) ecotypes that were located at the separated branches of two separate sub-clusters (Fig. 3).

Discussion

The comprehensive genetic characterization of indigenous chicken populations across diverse regions is crucial for the conservation and sustainable use of these valuable animal genetic resources. The use of the highly polymorphic microsatellite marker LEI0258 is

particularly valuable in this context, as it can provide insights into both the genetic diversity and potential immunity-related characteristics of the studied chicken populations (Lwelamira *et al.*, 2008; Owen *et al.*, 2008; Chang *et al.*, 2012; Mpenda *et al.*, 2020). It has been shown to be associated with both genetic diversity and immune-related traits in chickens (Nikbakht and Esmailnejad, 2015; Esmailnejad *et al.*, 2017). This study provides valuable insights into the genetic diversity of various chicken populations from Iran, Indonesia, Nigeria, and Tanzania, as revealed by the analysis of the highly polymorphic microsatellite marker LEI0258.

The Iranian chicken populations exhibited a wide range of genetic diversity. The local ecotypes, displayed higher effective number of alleles and observed heterozygosity as compared with the commercial Ross 308 breed (Table 2). This suggests that the indigenous Iranian chicken populations harbor substantial genetic resources that could be of interest for conservation and breeding programs.

In comparison with other reports, the number of alleles found in different populations varies, ranging from 39 in Nigerian chicken, 25 in both Iranian and Indonesian chickens, 23 alleles in Tanzanian ecotypes, 15 in Brazilian chickens, and 26 in Vietnamese and North American/European chickens (Lima-Rosa *et al.*, 2005; Mwambene *et al.*, 2019; Olufowobi *et al.*, 2020; Mustofa *et al.*, 2022). In addition, a comparison of the specific LEI0258 alleles of Iranian chicken with other regions indicated that 9 out of 25 alleles (i.e. 182, 194, 223, 230, 247, 487, 513, 539, and 552) were unique to the Iranian chicken populations tested. Interestingly, none of the seven Ross 308 alleles were found in Iranian chicken ecotypes.

The Mazandaran chicken ecotype displayed the highest effective number of alleles (Ne=16.619) and Nei's diversity index (Nei=0.939), indicating an exceptional level of genetic diversity. In contrast, the commercial Ross 308 broiler exhibited the lowest effective number of alleles (Ne=4.845) and Nei's diversity index (Nei=0.794), suggesting a relatively lower level of genetic diversity. The Ross 308 broiler line had the highest observed heterozygosity (Obs_Het=97%), indicating high levels of genetic admixture and outcrossing. Conversely, the Arian indigenous population had the lowest observed heterozygosity (Obs_Het=34.5%), which may be attributed to higher levels of inbreeding or population substructure. The deviations from Hardy-Weinberg equilibrium (HWE) observed in the Ross 308, Arian, and Khorasan populations suggest the influence of non-random mating, selection, mutation, or gene flow. Conversely, Marandi, Mazandaran, Uremia, and Esfahan populations were in HWE, indicating random mating and evolutionary equilibrium.

In the context of indigenous chicken populations, evaluating PIC of highly polymorphic markers, such as the LEI0258 locus, provides further insights into the informativeness of the genetic markers used, and the relative levels of genetic diversity. Accordingly, the

Esfahan population had the highest PIC (0.946), and the Khorasan population had the lowest PIC (0.107). The other indigenous populations, such as Arian (PIC=0.772), Marandi (PIC=0.895), and Mazandaran (PIC=0.895), displayed high PIC values, highlighting their genetic wealth and the potential of the marker for diversity assessments. These data suggests that indigenous populations harbor unique allelic variations that could be leveraged for the development of locally adapted chicken varieties. The PIC value observed in the Nigerian indigenous chicken was also exceptionally high (0.981) (Olufowobi *et al*., 2020) while the Tanzanian ecotypes displayed a very high PIC value (0.935) (Mwambene *et al*., 2019) and the Indonesian chickens had a slightly lower PIC value (0.811) (Mustofa *et al*., 2022). The particularly high PIC values in Nigeria and Tanzania suggest that these regions may be genetic diversity hotspots for chickens.

Genetic diversity analysis indicated that variations partitioned into two components: among populations (13.34%) and within populations or individual variations (86.66%). These values were statistically significant (P<0.001), indicating that there is a significant genetic structure among the populations that are not completely homogeneous and can be considered as distinct genetic resources. In comparison between countries, the genetic differentiation among populations was higher than the values reported for the Nigerian (2.7%) and Tanzanian (4.8%) chicken populations. Moreover, the genetic diversity in the Nigerian (97.7%) and Tanzanian (95.2%) chicken populations is more concentrated within the populations, as compared with the populations analyzed in this study. This suggests that the Iranian ecotypes are more genetically structured and differentiated as compared with the Nigerian and Tanzanian populations.

The phylogenetic tree shows the evolutionary relationships among different chicken populations from Iran (Fig. 2). Based on the results, the Ross 308 population appears to be the most genetically distinct from the rest, as it branches out earliest from the common root. Among the Iranian ecotypes the chickens were sub-clustered according to their geographical origin except for Marandi and Mazandaran that seems to have the same origin, suggesting they are more closely related to each other as compared with the other populations (Fig. 2). The tree provides insights into the evolutionary relationships and genetic differentiation among the Iranian chicken populations included in the analysis.

This phylogenetic tree for different countries, including Nigeria, Indonesia and Tanzania shows clear clustering of populations based on their geographic origins (Fig. 3). According to the distinct clustering, it was likely that the Iranian chicken ecotypes had influenced very different evolutionary history from the other populations. The Nigerian populations form a well-defined cluster, with Dourgou, Goggori, Tchagara, Kolonto, and Popular forming a tight sub-cluster. The Merawang and Pelung populations, from Indonesia, also cluster closely with the Nigerian populations, indicating some genetic similarities. As presented, one of the

Iranian ecotypes (Arian) showed a more compact association with chickens from Indonesia (Black Kedu) rather than other populations studied (Fig. 3). The Tanzanian populations form a separate cluster, with Chunya, Mbarali, Namtumbo, Ileje, Wanging-ombe, Itunduma, Msimbazi, Mbeya, Njombe, and Songea, suggesting that the Tanzanian chicken populations are genetically distinct from the Nigerian and Iranian populations (Fig. 3). Populations within the same geographic region tend to be more closely related, while the differences between the broad geographic regions are more pronounced. The genetic distances between the Nigerian, Tanzanian, and Iranian populations suggest that they have experienced distinct evolutionary trajectories and have accumulated genetic differences over time.

The high level of polymorphism exhibited by LEI0258 across the different chicken populations examined, with up to 80 alleles detected, underscores its use as an informative marker for assessing the genetic resources of indigenous breeds. Furthermore, the LEI0258 locus has been linked to various immune response-related genes, suggesting that the genetic diversity observed at this marker may also reflect differences in the immunocompetence of the studied chicken populations. Polymorphism in alleles related to immune responses is of great importance from two aspects. With the presence of diverse alleles in the population, the selection of lines or strains resistant to diseases becomes more likely, which can be achieved through breeding preprograms. On the other hand, as previously indicated, some LEI0258 microsatellite alleles are associated with a stronger immune response to common vaccines, which can lead to populations that require fewer vaccinations and even use these findings for vaccine design and production (Nikbakht and Esmailnejad, 2015; Esmailnejad *et al*., 2017). This dual perspective, encompassing both neutral genetic diversity and potential functional implications, highlights the value of using markers like LEI0258 in comprehensive genetic characterization studies of indigenous chicken resources.

Acknowledgement

Conflict of interest

The authors declare that they have no conflict of interest.

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