Immunogenetics and genetic variations in indigenous chicken in the tropics using SNP data

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Abstract
This study used data from 150 indigenous chicken from four agro-ecological zones in Rwanda to provide deep insight of the population structure and variation of the immunogenetics using several approaches based on phenotypic and SNP data. The population structure of indigenous chicken was analysed using Principal Component Analysis (PCA), ADMIXTURE analysis, and phylogenetic relationships for the whole genome and at chromosome 16. The study used 65,945 SNPs from the collected chicken. Phenotypic analysis was done for the Newcastle disease titer (ab) alongside bodyweight at 20 weeks with the highest having 1.6kg. The genome analysis was done using the genotyping-by-sequencing approach. The grouped the indigenous chicken into two genetic clusters, which was confirmed by ADMIXTURE analysis that revealed that the lowest cross-validation (CV) error (0.51) was at K = 2. The analysis of Population structure at chromosome 16 showed that the population had the lowest CV error (0.50) at K = 1. The mean body weight and antibody titer were 1673.61 ±237.14g and .5±55.35, 1311.34±121.9g and 8832.5±55.36, respectively, depicting an inverse relationship between bodyweight and antibody titers. The cluster means for body weight and antibody titers were significantly different (P < 0.001) for body weight and antibody titers. The indigenous chicken genetic clusters in Rwanda have variation in antibody titers which can be attributed to varied selection pressure. The observed genetic diversity of the indigenous chicken for disease resistance should be well-thought-out when scheming a selection programme to ensure that the ICs population is sustainable, flexible and simultaneous improvement of this trait. Based on this study’s findings government should implement strategies that conserve and maintain the genomic diversity of Rwanda indigenous chicken.

Keywords: Chromosome 16, immune traits, flexibility, MHC, sustainability

1. Introduction
Indigenous Chicken farming has been gradually shifting from subsistence to commercial chicken farming due to increased demand for IC meat and eggs (Magothe et al., 2019) [25]. The increase in IC demand makes farmers house birds in large flocks at high stocking density thus the increased risk of diseases, and disease spreading (Mujumbere et al., 2022) [26]. Newcastle disease is one of the common diseases affecting IC farming (Kapczynski et al., 2013; Walugembe et al., 2019) [20, 43]. Identifying the genes that control disease resistance would make the selection of IC for improved productive performance and enhanced disease resistance possible. A useful spinoff would reduce cost of production due to decreased use of drugs, as well as better product quality due to lowered drug residues (Jie & Liu, 2011; Dar et al., 2018) [19, 9].

A number of efforts have been practiced worldwide to appreciate and improve resistance to disease in livestock through the application of immunogenetics. In pigs improvement of resistance to disease was applied using gram–negative bacteria (Zhao & Chen, 2012) [40] and in ruminants it was done using gastrointestinal nematodes (Sweeney & Good, 2016) [30]. In bovine, immunogenetics was applied to improve resistance to mastitis (sodeland et al., 2011) [39]. Information on both immunology and genetics of animal would well describe the disease phenotype (Bishop, 2014) [6]. Immune capability related to a particular disease used can be to show indirect selection for resistance to disease because these traits can be assessed and measured in breathing animals (Luo et al., 2013) [22]. Santos-Argumedo, 2012 showed that antibody titers are immuno logical traits which can be inherited in poultry thus making it easy to determine loci or a particular gene associated to immune-related traits.
In chicken, selecting for growth and production traits has been related with decrease in immunity (Bayyari et al., 1997; Wondmeneh et al., 2015) [6, 40]. Selecting chicken with increased weight of the body is genetically related to a reduction in disease resistance. Genetic correlations between BW & AB in chicken (\(r_c\)) are medium to high (Mebratie et al., 2019). Using microsatellites, Ngeno et al., (2015) and Habimana et al. (2022) [10] showed genetic structuring in IC in Kenya and Rwanda. The studies reported that body weight was inversely related to Abs. Microsatellites are more variable but suffer from ascertainment bias, homoplasy and amplification variation of primers (Tian et al., 2008) [41]. High-density single nucleotide polymorphism has made it possible in the investigation of the population’s genetic structure through use of large numbers of markers and identify regions in the genome where events related to the traits we are interested with (Groenen et al., 2008; Wollstein et al., 2010) [17, 48]. SNPs are more abundant and evenly distributed across the genome hence more informative. Therefore, the study aimed to examine the population structure of ICs at both whole-genome and chromosome 16.

Materials and Methods

The study population and housing

A total 150 IC were sampled randomly from the northern, southern, central, and eastern agro-ecological zones of Rwanda. The indigenous chickens were kept at the University of Rwanda under the same environmental conditions under deep litter system. The standard density of 12birds/M², 22-23 hours of light was provided. Infrared heat lamps of 250 Watts were used to heat the house. Afterward, 23-25°C of temperature was provided during the entire study period.

Collection of phenotypic data

The indigenous chickens were vaccinated with two commercial New castle Disease virus live vaccines. At 2 days of age, AVI New castle Disease HB1 was put in water and the second shots of the vaccine (AVI ND Lasota) were given on their eye when they were at 28 days old. On the 35th day, collection of blood was done without anticoagulant for separation of serum for detection of antibody titers against New castle disease vaccine. The detection of antibody responses to New castle disease was using an indirect ELISA test. The Antibody levels were computed using the ID Soft TM data analysis programme. At the 20th week of age, the body weights of the IC were measured.

Extraction of genomic DNA and genotyping

Blood samples for DNA extraction were collected using 2.5ml EDTA tubes. Genomic DNA from blood was extracted by a DNA extraction kit. The concentration of extracted genomic DNA and the qualities were evaluated using a Nano Drop™ 2000 spectrophotometer (Thermo Scientific™ Nanodrop 2000) and gel electrophoresis (1% agarose) (Lu, et al., 2016) [25]. Raw reads were obtained using the Genotyping-by-sequencing (GBS) approach (Jain et al., 2016) [24].

Alignment of the reads and calling of the SNPs

Trimming of the raw reads was done using the sickle tool and then they were aligned to the Galgal4 chicken reference genome using Burrows- Alignment tool (BWA v0.7.17), afterward sorting of the reads was done. Removal of the duplicated reads was performed using SAM tools v1.3.1. The calling of SNPs was done using SAM tools v1.3.1.

Quality control of the SNPs

Trimming of the reads was done using a sickle and then alignment was done by aligning the raw reads to the Galgal4 (chicken reference genome) by the Burrows-Alignment tool (BWA v0.7.17). The removal of duplicated raw reads and calling of the reads was performed using the SAMTools v1.3.1 (Li et al., 2008). The SNPs obtained were thereafter subjected to the standard filtering procedures using Plink v1.07 software (Purcell et al., 2007); minimum SNP quality of 20, 5% missing SNP genotypes, Hardy–Weinberg equilibrium (\(P<10^{-6}\)), call rate > 95%, heterozygosity > 0.4, and minor allele frequency > 0.03. Pairwise linkage disequilibrium (LD) measured by \(r^2\) values for each chromosome (Bradbury et al., 2007) [7] was calculated using Tassel 5.2.60.

Statistical analysis

Phenotypic characterization

Data collected on antibody titers against Newcastle disease and BW of ICs populations were entered into a database using Microsoft Office Excel 2016. The phenotypes were USED to place the chicken in unique clusters based on either AB, BW and AB and BW using PROC FASTCLUS in SAS software v 9.4 (2008). The within-cluster variation was described using descriptive statistics. The PROC GLM of SAS was used to determine whether the clusters were differing significantly for AB and BW based on the different clustering approaches. The following linear model was used:

\[
y_{ij} = \mu + t_i + e_{ij}
\]

Where

\(y_{ij}\) is the total IgY titer or body weight; \(\mu\) is the overall mean; \(t_i\) is the effects of the fixed factor and \(e_{ij}\) is the residual term.

Genotypic clustering of IC into genomic clusters

The SNPs genotypes of the IC were used to assess the genetic differentiation of the IC population. The study used PCA to minimize the dimensionality of the SNP data set with a large number of interrelated variables. This was achieved by converting the original variables into a new set of variables, the principal components (PCs), which were uncorrelated, and ordered so that the first few retain most of the variation present in all of the original variables. The genomic clusters were determined by plotting PC1 and PC2 in tassel software.

Admixtural analysis

ADMuXTURE was to determine population relatedness and assigns populations to ancestral clusters. Population structure was examined investigation of the population structure was done using the model-based clustering algorithm that was run in ADMIXTURE (Alexander et al., 2011) [2], from K = 2 to 4. The cross-validation method was used in estimation number of populations that are most likely to be found. The K value reduces the cross-validation prediction error was then assumed as the most likely (Evanno et al., 2005) [12]. The ip ADMIXTURE R package was used to represent the results in a graph (Amornbunchornvej et al., 2020) [3].

Estimation of genetic differentiation

The unbiased genetic differentiation estimate, \(F_{ST}\) (Weir & Cockerham, 1984) [44] was calculated using admixture software in Kenet vLab (a virtual software) with the quality-controlled SNP dataset to estimate genetic differentiation between populations using the fixation index.

Phylogenetic analysis

The relationship among IC from the four agro ecological zones was determined by constructing a matrix distance, from
the matrix distance a phylogenetic tree was drawn in Tassel software (Bradbury et al., 2007) [7]. The genetic relationship between the IC from the four zones was determined based on the neighbour-joining tree algorithm in TASSEL software v5.2.35 (Bradbury et al., 2007) [7]. The neighbour-joining tree cladogram generated by TASSEL was visualized in the archaeopteryx tree (Bradbury et al., 2007) [7].

Genetic diversity at chromosome 16

The study extracted SNPs from chromosome 16 (position) using 90 SNPs that covered from LOC425771 through CD1A1 (210.000 bp) (Fulton et al., 2016) [14]. The extraction was done using VCF tools v0.1.14 (Danecek et al., 2011) [10]. Diversity was done following steps described above that is i) PCA ii) admixture analysis and iii) Phylogenetic relationship that is the neighbour-joining analysis. The SNP allele frequencies for chromosome 16, expected (He) and observed (Ho) heterozygosity and hardy Weinberg equilibrium (HWE) were computed using PLINK (v1.90b) software (Slifer, 2018) [40].

Results

Phenotypic clustering: Phenotypic clustering indicated that the Rwanda indigenous chicken were clustered into two populations. Cluster one mean body weight of 1673.61 ±237.14g and antibody titer were 4912.5±55.5. Cluster 2 had mean body weight of 1311.34±121.9g and mean antibody titer of 8832.5±55.36. The clusters differed significantly (P < 0.001) for body weight and antibody titer. The cluster with high mean in bodyweight and low mean in titer and vice versa.

The analysis of Population structure

This was done using the principal component analysis (PCA), admixture and neighbour-joining tree analyses. This analysis was done at the whole-genome and chromosome 16.

Population structure analysis at whole genome

At the whole genome level, PCA showed that principal component one (PC1) amounted to 38% and principal component two (PC2) amounted to 26% of the total variability (Figure 1 and 2).

![Fig 1: Plot of principal component analysis in tassel software showing the two genetic clusters of indigenous chicken from the four ecological zones.](image-url)
Admixture analysis

Admixture analysis performs maximum probability approximation of individual ancestries from multilocus SNP genotype data. A R package, ipADMIXTURE (Amornbunchornvej et al., 2020) was then used to plot the ancestry of the indigenous chicken from the four zones. Admixture plots arranged the individuals according to the portion of origin they shared with other individuals. The Bayesian clustering analysis of ADMIXTURE resulted to K values from K2 to K4 as in Figure 4, and K2 was found to be with the lowest cross-validation error (Fig. 3). When K was 2 the indigenous chicken from the four agro-ecological zones were totally admixed with the majority being almost pure. When K was 3, IC from northern were pure. When the K value was 4, central and S (southern) showed that the IC were pure breed.

Fig 2: Principal component analysis of the whole genome showing two clusters, one cluster seemed to be the outliers form of the other cluster.

Fig 3: The cross-validation errors of the k values
The analysis Population differentiation

The magnitude of population differentiation between different IC from the four agro-ecological zones was investigated by using $F_{ST}$ values which were calculated using genotype data that was filtered (Table 1). For the whole population $F_{ST}$ values ranged from 0.071 to 0.218, indicating genetic differentiation appeared between IC from the four regions. However, these $F_{ST}$ values seem to increase. For instance, the $F_{ST}$ value was 0.092 between northern and southern and increased to 0.186 between northern and central zones. The opposite was observed between southern vs central (0.176) and southern vs eastern (0.071).

Table 1: Fixation index ($F_{st}$) of the population from the four agro-ecological zones

|                | Northern | Southern | Central | Eastern |
|----------------|----------|----------|---------|---------|
| Northern       |          |          |         |         |
| southern       | 0.092    |          |         |         |
| central        | 0.186    | 0.176    |         |         |
| eastern        | 0.140    | 0.071    | 0.218   |         |

Population structure analysis at chromosome 16

Twenty variants were extracted from chromosome 16. The variants were retained for downstream analysis that is, PCA, admixture analysis and neighbor joining analysis. The PCA and admixture analysis placed the indigenous chicken as one population as in (figures 5, 6, 7). Admixture analysis revealed lowest cross-validation error when $K$ was 1.
Phylogenetic relationship

The chromosome 16-based SNP phylogenetic analysis was used to deduce the relationships between indigenous chicken collected from four agro-ecological zones in Rwanda. The phylogenetic tree, together with the details on each indigenous chicken is shown as in Figure 7. The neighbour-joining tree reveals all the indigenous collected from the four zones had one common origin thus grouping them as one population.

![Figure 6: Principal component analysis of chromosome 16 showing one cluster of indigenous chicken from the four agro-ecological zones](image)

![Figure 7: Neighbour-joining tree obtained from the distance matrix of tassel software among 142 indigenous chicken from the four agro-ecological zones; this tree was only for 20 variants extracted from chromosome 16.](image)
Admixture analysis at chromosome 16
The Bayesian clustering analysis of ADMIXTURE resulted to K values from K1 to K4 and K1 was found to be with the lowest cross-validation error (Figure 8). This shows that at chromosome 16 level the indigenous chicken were grouped as one population.

![Fig 8: showing the cross-validation errors of the k-clusters](image)

Basic Genetic Parameters of a Population
Observed heterozygosity (Ho) values averaged 17.55±0.12 across all the IC groups from the four agro-ecological zone. The expected heterozygosity values averaged 15.77 ± 0.02 across the four populations. The heterozygosity rate ranged from 0-0.3 across the populations, with 30 individuals having more than 0.2 heterozygosity rate as in Table 2. The average inbreeding coefficient per population was 0.43±0.03 across the four populations. The inbreeding coefficients per agro-ecological zones were as follows Central had the highest with 0.5 followed by eastern with 0.4 then northern with 0.4 and lastly southern with 0.3.

| CHR | SNP | AI  | A2  | MAF  | NCHROBS |
|-----|-----|-----|-----|------|---------|
| 16  | 100134902| F|0-45:G>A-45:G>A | G  | A       | 0.4615  | 286 |
| 16  | 100149083| F|0-10:G>A-10:G>A | G  | A       | 0.3169  | 284 |
| 16  | 100129173| F|0-17:C>A-17:C>A | A  | C       | 0.003497| 286 |
| 16  | 100034475| F|0-22:C>T-22:C>T | T  | C       | 0.006993| 286 |
| 16  | 100103111| F|0-39:T>C-39:T>C | C  | T       | 0.003497| 286 |
| 16  | 100094740| F|0-25:T>G-25:T>G | G  | T       | 0.01748 | 286 |
| 16  | 100085316| F|0-26:T>A-26:T>A | T  | A       | 0.465   | 286 |
| 16  | 100088251| F|0-14:A>G-14:A>G | A  | G       | 0.01748 | 286 |
| 16  | 100163669| F|0-42:G>T-42:G>T | G  | T       | 0.0979  | 286 |
| 16  | 100163035| F|0-13:G>A-13:G>A | A  | G       | 0.0461  | 282 |
| 16  | 100125696| F|0-68:C>G-68:C>G | C  | G       | 0.08741 | 286 |
| 16  | 100096683| F|0-36:G>T-36:G>T | T  | G       | 0.1119  | 286 |
| 16  | 100021966| F|0-8:G>A-8:G>A | A  | G       | 0.003497| 286 |
| 16  | 100006068| F|0-32:A>G-32:A>G | G  | A       | 0.09991 | 286 |
| 16  | 100077597| F|0-8:A>G-8:A>G | G  | A       | 0.3741  | 286 |
| 16  | 100130892| F|0-21:T>C-21:T>C | C  | T       | 0.3636  | 286 |
| 16  | 100147497| F|0-22:G>A-22:G>A | A  | G       | 0.01049 | 286 |
| 16  | 100118266| F|0-26:C>G-26:C>G | C  | G       | 0.5     | 266 |
| 16  | 100125885| F|0-14:A>G-14:A>G | A  | G       | 0.01399 | 286 |
| 16  | 100033338| F|0-31:C>A-31:C>A | A  | C       | 0.243   | 284 |

SNP Marker Characteristics
Minor allele frequency (MAF) had an average of 0.16176 and SNP regions 100163035|F|0-13:G>A to 100021966|F|0-8:G>A-8:G>A had a MAF value that was below 0.05. An analysis of how the minor allele frequency were distributed across the 20 variants revealed that over 50% of the markers were within the 0–10% minor allele frequency threshold.

| Table 2: heterozygosity rates across the four agro ecological zones |
|---------------------------------------------------------------|
| Heterozygosity rate | 0 | 0.10 | 0.05 | 0.15 | 0.20 | 0.21 | 0.25 | 0.26 | 0.30 |
| No of Indv | 18 | 51 | 40 | 18 | 2 | 6 | 1 | 3 |

Discussion
This study focused on phenotypic and genomic characterization (at both whole genome and at chromosome level) of IC in Rwanda. The findings revealed both
phenotypic and genotypic characterization at whole genome grouped the IC into two groups providing a deeper understanding of the structure of IC population to supplement the use of phenotypes for IC selection (Chiwanga et al., 2020). At chromosome level (chromosome 16) and the whole genome, the genetic diversity analysis revealed one and two genetic groups, respectively. These results indicate that at chromosome level, that there maybe significantly less genetic variation than the variation within the whole genome of a population.

**Phenotypic clustering**

Regardless of extensive information of the genomic foundations of phenotypes the G. gallus, few and non-exhaustive studies have measured genotype and phenotype variations in populations of this species. In this study, measurements of antibody titers and Body weight were evaluated and compared among the IC obtained from the four agro-ecological zones. Phenotypic clustering grouped the IC into two clusters. The first cluster had a high mean body weight and low antibody titers as compared to cluster two which had a low mean body weight and high mean for antibody titers. Selecting for growth and production traits has been linked with reduced immunity. Selection for increased BW has been shown to be genetically associated with a reduction in disease resistance in chicken. (Bayyari et al., 1997; Wondmeneh et al., 2015) [4, 46]. This study’s findings revealed that IC with high body weight have reduced antibody titers and vice versa when they have high antibody titers their body weight is reduced.

**Genetic diversity at whole genome**

The study reports the diversity of the genome of Rwanda IC through identifying and characterizing of 65,945 SNPs from 142 IC obtained from four agro-ecological zones that are northern, eastern, southern and central using whole-genome. The analyses of the principal component and admixture revealed two ancestral gene pools across the Rwandan IC populations. A similar study done in Tanzania to investigate the population structure of Indigenous chickens using admixture grouped the selected IC into two gene pools (Mushi et al., 2020) [27]. In agreement with the Principal Component Analysis results, admixture analysis grouped all the indigenous chicken from the four agro-ecological zones into into two unlike a study done by Habiminana et al. (2020) which revealed four gene pools using microsatellite. When K=2 the Rwandan indigenous chicken were grouped into central and Eastern, with more proportion of the central ancestry cluster probably because of interbreeding among the IC (; Mushi et al., 2020) [27]. When K = 3, all samples were grouped into three clusters, each for pure IC from central and admixed. At K= 3, the IC from northern and central presented a homogenous cluster (Black and light blue respectively) which was not displayed by ICs from eastern and southern agro-ecological zones. In overall, the IC population were admixed with most birds being from the central agro ecological zone. Our results revealed that the Fst values for the whole population ranged from 0.071 to 0.218 thus indicating isolation between the IC populations, and this mightly likely mean that the IC populations are presently breeding with one another. Based on this study’s findings, government should implement strategies that would conserve Rwanda indigenous chicken genetic diversity and its characteristics.

**Genetic diversity at chromosome 16(MHC region)**

At chromosome 16, the investigation of the population structure by Principal Component, admixture analysis and neighbour joining approaches grouped the Rwanda indigenous chicken as one population. The means that there is horizontal gene flow probably due to small geographical size of the country. A study done in Kenya using microsatellite grouped IC based on MHC-linked markers with grouped Indigenous Chickens into three groups, composed of birds from dissimilar ecotypes while clustering based on non-MHC markers placed indigenous chicken into two gene pools (Ngeno et al., 2015). The absence of geographical boundaries, the purchasing of the seeder flocks from one geographical location to another and the free-range system of chicken management in the tropics might be the reason of increase in the interbreeding among the IC resulting in one Rwanda IC population. Common cultural practices like intermarriages among the tribes might have attributed to recurrent flow of gene in the locations. Equally, interactions of humans among countries like Kenya through trade lead to mixing up of IC (Mwacharo et al., 2013b; Mushi et al., 2020) [29, 27]. Chromosome 16 was analyzed because is houses MHC. Studies have reported that MHC is related with disease resistance and immune traits (Fulton et al., 2016, 2017) [14]. Heterozygosity can also be used in the analysis of diversity of a population genetics. The mean of heterozygosity of a population indicates the level of its constancy. When the heterozygosity of a population is low, this notifies there is a high genetic constancy (Cheng, 2010) [9]. This study revealed that observed heterozygosity of the Indigenous Chicken population from the four agro ecological zones was ranging from 0 to 0.44 with an average of 0.11, while expected heterozygosity ranged from 0.007 to 0.5 with mean of 0.21. This study also revealed that the value of observed heterozygosity was lower than expected heterozygosity thus this could be attributed to forces like inbreeding and the F estimates (0.43). In this study classification according to immunological traits based on Newcastle disease antibody titers revealed selection pressure of indigenous chicken in indigenous chicken in Rwanda.

**Conclusions**

Phenotypic and genotypic clustering found 2 gene pools of IC in Rwanda. This shows that phenotype can also be used in the identification of different chicken ecotypes. Based on population structure analysis using SNPs the Rwanda Indigenous chicken belong to two genetically different groups at the whole genome. Population structure analysis at chromosome 16, placed the IC population as one cluter. The observed genetic diversity of the indigenous chicken for disease resistance should be well-thought-out when scheming a selection programme to ensure that the ICs population is sustainable, flexible and simultaneous improvement of this trait. Based on this study’s findings government should implement strategies that conserve and maintain the genomic diversity of Rwanda indigenous chicken.

**Ethics approval**

This study used indigenous chicken to collect the data and the study was approved by the ethical committee of Egerton University, Nakuru, Kenya.

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Declaration of interest
The authors declare no conflict of interest

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