Genetic variation of LEI0258 locus at major histocompatibility complex (MHC) region in Ethiopian indigenous village chicken

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ABSTRACT

Indigenous chickens are locally adapted to environmental challenges and provide subsistence to millions of farmers in Africa. However, their productivity remains low compared to exotic strains. Efforts are being made to combine the local adaptation of indigenous chicken with productive traits of exotic chicken. Understanding the link between genetic diversity and environmental challenges leads to marker-assisted breed improvement programs for sustainable chicken production at smallholder level. Genetic variation at LEI0258 VNTR locus located within the MHC region has been linked to infectious disease resistance/susceptibility in commercial breeds. The aim of this study was to investigate allelic variability, genetic diversity and genetic relationships of 24 chicken populations in Ethiopia. Here, the diversity at LEI0258 in 236 chickens from 24 Ethiopian indigenous chicken populations using the major Histo-compatibility Complex linked LEI0258 marker is reported. A total of 236 DNA samples were genotyped by capillary electrophoresis from 24 chicken populations. The number of alleles, allele frequency, and heterozygosity levels were used to measure genetic variation at LEI0258 locus in Ethiopian indigenous village chicken. Twenty-nine LEI0258 alleles were observed using capillary electrophoresis that ranged from 185 to 569 bp with no significant difference in allele frequencies between populations. The number of alleles ranged from 179 (Meseret) to 569 (Batambie), with an average of 9.6 alleles per population. Allelic polymorphism was further evaluated through genotyping by Sanger sequencing. Twenty-three DNA samples with different fragment sizes were re-amplified and their alleles sequenced to depict polympormisms based on the combination of two repeat regions at 12 bp and 13 bp, respectively, and flanking regions with SNP and indels. The repeat region at 12 bp appeared 2 to 18 times, whereas the region at 13 bp appeared invariant in all populations. Sequence relationships revealed two distinct groups of alleles. The number of indels and mutations were 33 and 17, respectively. From capillary electrophoresis, the fixation coefficient of the sub-population within the total population (F_{ST}), inbreeding/fixation/ coefficient of an individual in a sub population (F_{IS}) and total inbreeding /heterozygosity deficit/ coefficient of an individual within the total population (F_{IT}) in the locus was 0.03, 0.08 and 0.11, respectively. Three percent of the genetic diversity was due to differences among populations, where as 8% and 89% were variations among individuals and variations within individuals, respectively. Despite the overall low genetic differentiation, both fragment and sequencing analysis revealed high allelic and genetic variability across the 24 populations. The high diversity at LEI0258 in Ethiopian indigenous village chicken populations supports the importance of the MHC region in relation to the disease challenges faced by smallholder poultry farmers across Ethiopia. We recommend that breed improvement programs ensure the maintenance of this diversity by selecting breeding stock as diverse as possible at the LEI0258 locus.

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INTRODUCTION

High evolutionary pressures occurred in chicken during the course of domestication and subsequent natural and human selection (Downing *et al.*, 2009). Among others, infectious diseases exert strong selective pressure by affecting genes associated with innate and adaptive disease resistance and susceptibility. According to Salomonson *et al*. (2014) many genes involved in immunity are part of multigene families. In some families, each gene is conserved for a specific function dedicated to a particular outcome, in others allelic polymorphisms and copy number variations allow rapid evolution in response to new environmental challenges, and there are also families comprising of both kinds of genes. The chicken Major Histocompatibility Complex (MHC) is one of these multigene families comprising loci encoding receptors which bind amino acid fragments from foreign pathogens on the surfaces of various immune and non-immune cells (Jarosinski *et al*., 2010; Chen *et al.*, 2012; Nikbakht *et al.*, 2013; Keambou *et al*., 2014; Ncube *et al.*, 2014; Fulton *et al.*, 2016a). MHC is a cluster of over 80 genes (92 kb) spanning chromosome 16 (Chazara *et al.*, 2011, 2013; Walker *et al*., 2011; Nikbakht and Esmailnejad, 2015; Miller and Taylor, 2016) characterized by high polymorphism and a tight linkage into a single supergene complex, which are believed in association with disease resistance or susceptibility.

LEI0258 is a highly polymorphic variable number tandem repeat (VNTR) located near the BL/BF region of MHC-B locus on chromosome 16 with over 35 alleles (Fulton *et al.*, 2016a; Kannak *et al*., 2017). This marker has been utilized as indicator of MHC diversity due to the low cost, easy access to detection technology and rapid results. The length variations of the LEI0258 are large and discrete; many alleles can be distinguished through agarose gel electrophoresis (Fulton *et al*., 2016a). Although it is considered valuable, fewer limitations have been reported by different works like variation in instrumentation that can result in small size differences (1-6 bp). Similarly, different MHC-B serological defined and SNP defined haplotypes can have the same LEI0258 allele size (Fulton *et al*., 2006). Fulton *et al*. (2016a) also described that the use of this single marker is known to underestimate MHC variation owning a mutation rate of 0.11% and cannot detect the existence of MHC recombinants as it examines only one location of the MHC. However, currently typing the MHC based on the VNTR LEI0258 is the most used method to obtain genetic information on this region (Fulton *et al*., 2016b). It has been reported to be associated with chicken performance and disease tolerance (Nikbakht and Esmailnejad, 2015), including allelic variation in antibody responses to vaccination against Newcastle Disease Virus (NDV) (Nikbakht *et al*., 2013; Baelmans *et al.*, 2005), Marek's disease (Wang *et al.*, 2014; Fulton *et al*., 2016a), corona virus (Hateren *et al.*, 2013) and coccidiosis, as well as its association with body weight, survival, embryonic mortality, fertilization rate, hatchability, egg production and resistance to worms (Owen *et al.*, 2008). Given its high level of polymorphism and linkage disequilibrium with the MHC-B locus, LEI0258 genotypes have been

suggested as an indicator of MHC-B haplotypes, and it has become an important genetic marker used in chicken breed improvement programs (Hoque *et al*., 2011; Weigend *et al*., 2001; Banat et ak., 2013; Gao *et al*., 2015). This has been confirmed by Chazara *et al*. (2013) who has ascertained that the LEI0258 marker genotypes an excellent predictor of the heterozygosity at MHC locus.

LEI0258 is described as an atypical VNTR marker which is composed of 12 bp (CTTTCCTTCTTT) and 13 bp (CTATGTCTTCTTT) conserved repeat sequences which are flanked on both sides by indels and SNPs (Chazara *et al*., 2011; Lima-Rosa *et al*., 2005; Fulton *et al*., 2006;). In an association study of MHC haplotypes with Marek's disease, Bumstead (1998) found that 96.5% of the birds with B-21 haplotype were resistant to viral infection while birds with B-19 haplotype suffered 100% incidence of mortality. Fulton *et al*. (2006) observed allele size diversity ranging from 182 bp to 552 bp. Lwelamira (2008) genotyped two chicken ecotypes from Tanzania using LEI0258 marker and identified 23 alleles. They further reported that allele of 206 bp had significant positive correlation ($P < 0.001$) with elevated antibody responses against NDV vaccine, whereas allele of 307 bp was positively correlated with body weight trait.

Indigenous chickens (IC) (*Gallus gallus domesticus*) are widely distributed in the diverse agro-ecological zones of Ethiopia. Accordingly, they represent ecotypes which may possess unique combinations of alleles in a set of given gene (Ngeno *et al*., 2015). Relatively, few works have been done so far on the genetic characterization of Ethiopian indigenous chicken. In particular, no study has attempted so far to characterise the immune system of Ethiopian chicken. We report here the characterization and diversity of the MHC-linked LEI0258 VNTR marker in 236 indigenous chickens from 24 distinct populations sampled across Ethiopia.

MATERIALS AND METHODS

Whole blood sample collection

Study population

Sample size and sampling method

Blood samples were collected from 24 chicken populations in Ethiopia (Figure 1). Samples included 80 cocks and 156 hens. Except for the improved Horro, Gondar Zuria and Enderta populations, two villages per population were sampled (10 chickens from each village). One or two chickens were sampled per household. Improved Horro, the 8^{th} generation breeding stock, was sampled at Debre Zeit Agricultural Research Centre (EIAR). Sampling included chicken from different agro-ecological zones with altitude ranges of 730-3500 meters. From the wing vein

of each chicken, 50 - 250 µl of whole blood were drawn with syringes using cryo-tubes filled with 1.5 ml absolute ethanol (100%) following the guidelines available at https://www.sheffield.ac.uk/nbaf-s/protocols_list.

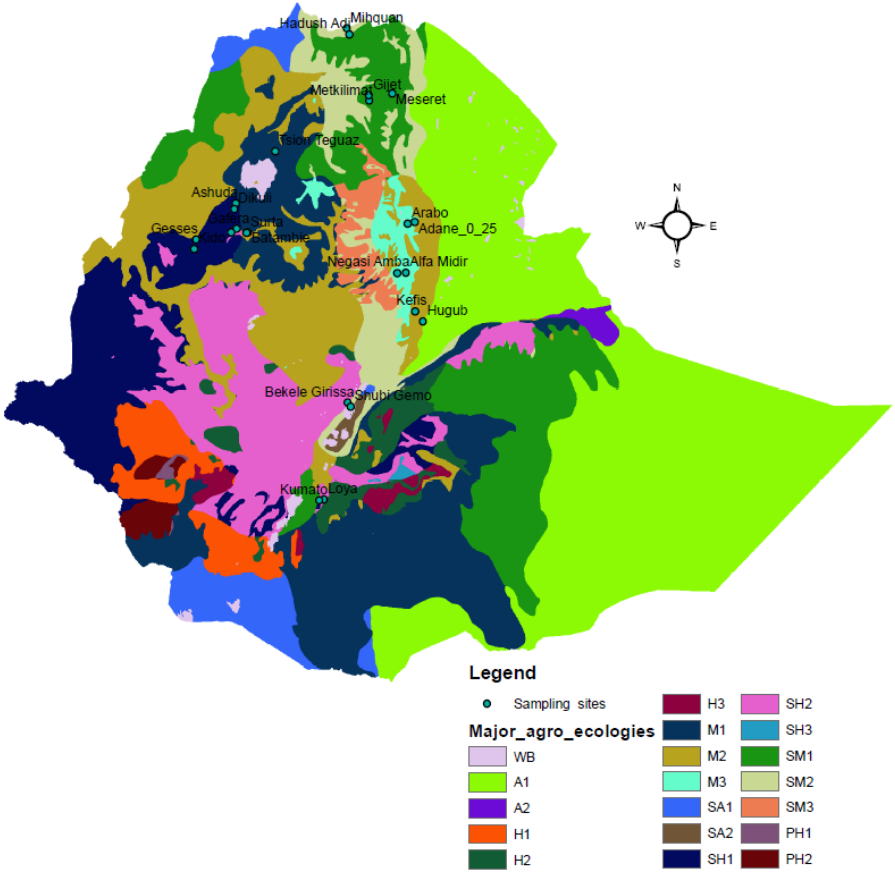

Figure 1. Agro-ecological map of Ethiopia with sampling site (MOA, 2000)

WB = Water body; A1 = Tepid to cool arid mid highlands; Hot to warm semi-arid lowlands; H1 = Hot to warm humid lowlands; H2 = Tepid to cool humid mid highlands; H3 = Cold to very cold humid sub afro-alpine to afroalpine; M1 = Hot to warm moist lowlands; m2 = Tepid to cool moist mid highlands; M3 = Cold to very cold moist sub-afro-alpine to afro-alpine; SA1 = Hot to warm semi-arid lowlands; SA2 = Tepid to cool semi-arid mid highlands; Sh1 = Hot to warm sub-humid lowlands; SH2 = Tepid to cool sub-humid mid highlands; SH3 = Cold to very cold sub-humid sub-afro-alpine to afro-alpine; SM1 = Hot to warm sub-moist lowlands; SM2 = Tepid to cool sub-moist mid-highlands; SM3 = Cold to very cold sub moist sub afro alpine to afro-alpine; PH1 = Hot to warm per humid lowlands; PH2 = Tepid to cool per-humid mid highlands.

Total DNA isolation

Total DNA was extracted from chicken whole blood at the BecA-ILRI Hub, Nairobi, Kenya facility (http://hub.africabiosciences.org/) using the Qiagen DNeasy blood and tissue kit protocol (Lwelamira *et al*., 2008). To evaluate the DNA concentration the Thermo Scientific NanoDrop spectrophotometer 2000c was used. The integrity of DNA was confirmed by agarose gel electrophoresis whereby 20 ng/µl genomic DNA samples were loaded with 1 µl loading dye (6X) on 1% agarose gel containing 2.5 µl gel red at a voltage of 7/cm for 60 minutes, 3 µl of lambda DNA of size of 48,500 bp and at concentration of 20 ng/µl were used as size marker. The gel was then examined using UV light by the GelDoc-It^2 Imager to check the DNA quality and quantity. The DNA was normalized to 20 ng/µl using milliQ water for polymerase chain reaction (PCR) and genotyping.

PCR amplification and genotyping using LEI0258 microsatellite marker

PCR amplification was carried out using the thermo-cycler PCR machine ABI PCR 9700 (Applied Biosystems). The primer sequences (GenBank accession number Z83781) for PCR amplification of LEI0258 were: forward 5'-CACGCAGCAGAACTTGGTAAGG -3' (length = 22 bp; GC content 47.6%; Tm = 71.5℃) and reverse-5'-AGCTGTGCTCAGTCCTCAGTGC-3' (length = 22 bp; GC content 46.2%; annealing temperature 69.9℃). The optimal PCR conditions were as described in (Gupta *et al*. (n.d); Han *et al*. (2013); Izadi *et al*. (2011); Nikbakht *et al*. (2013) either in a total volume of 10 µl including 2 µl of template genomic DNA (40 ng), 5 µl of non-dyed *Taq* DNA Polymerase (1000 U) (Shangai, China), 0.3 µl of PET-labelled forward primer (3 µM), 0.3 µl of reverse primer (3 µM) and 2.4 µl of milliQ water or in a total volume of 50 µl including 3 µl of 20 ng template genomic DNA, 25 µl of Dyed Bioneer Master mix (2x), 3 µl of forward primer (3 µM), 3 µl of reverse primer (3 µM) and 16 µl of milliQ water. The PCR conditions were set with an initial denaturation at 94℃ for 3 minutes; 30 cycles of 94℃ for 45 seconds, annealing temperature of 63℃ for 1 minute and extension of 72℃ for 1 minute; final extension at 72℃ for 20 minutes and final hold at 15℃. Two microliters of PCR product were loaded on a 2% agarose gel containing 2.5 µl of gel red and separated by electrophoresis at a voltage of 7/cm for 60 minutes. A 1 Kb ladder DNA from Bioneer was used as a reference to the size of the amplicons (Figure 2). The gel was exposed to UV light using GelDoc-It^2 Imager, to reveal the amplified fragments and their sizes.

For capillary electrophoresis, PCR product amplified using PET-labelled forward primer was added to a mixture of 12 µl GeneScan 500 LIZ® Size Standard and 1,000 µl of HIDI formamide, denatured at 95℃ for three minutes and separated by capillary electrophoresis using the ABI3730 DNA genetic analyzer (Applied Biosystems, Foster City, CA). The fragment (allele) sizes generated were scored with the GeneMapper Software Ver 4.1 (Applied Biosystems, Foster City, CA, USA)

and exported to Microsoft excel for preparation of input files for statistical analyses.

Sequencing for fine-analysis of fragment sizes

To confirm the polymorphisms from genotyping by capillary electrophoresis, PCR products of some homozygote and heterozygotes, were selected for the Sanger sequencing. The same primers used for genotyping were employed except that they were tailed with T7 (20 base pair) and SP6 (17 bp) primers for the forward and reverse primers, respectively. Homozygote DNA fragments were purified using GeneJet PCR purification Kit (Thermo Fisher Scientific; cat. No. K0701) while heterozygote DNA fragments were purified using the Qiagen Gel Extraction Kit and sent for sequencing to BIONEER sequencing platform in Korea. Alleles were sequenced on an ABI 3730XL DNA Analyzer using T-7 and SP6 primers. LEI0258 amplicons were sequenced in both directions as it provides confirmation of sequence and allow sequence information right up to the primer binding region.

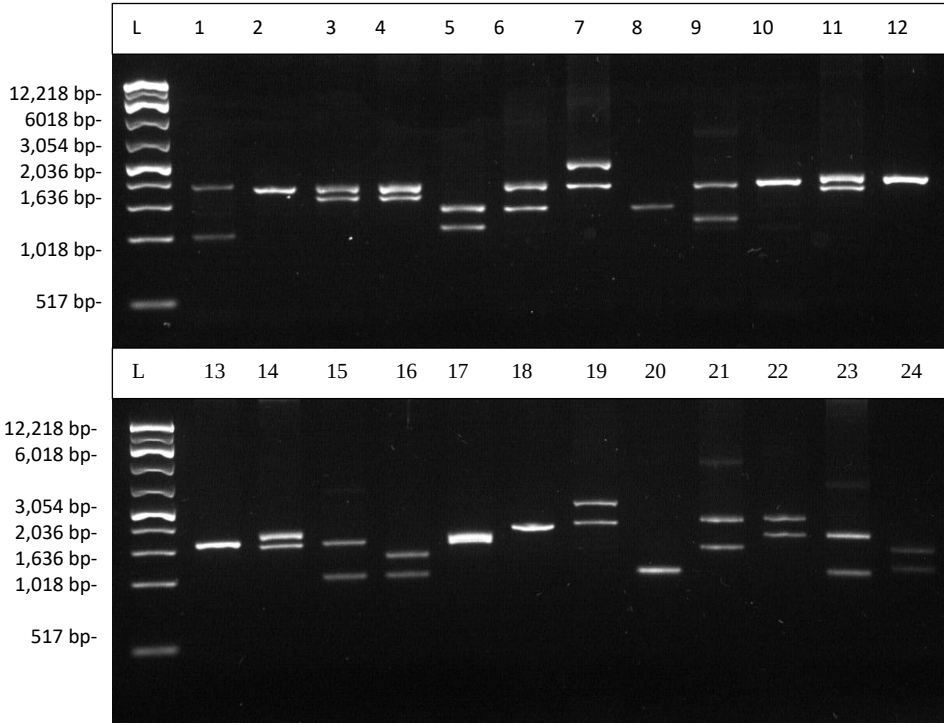

Figure 2. Image of alleles on 2% agarose gel run at 7/cm for 60 minutes in indigenous chicken populations of Ethiopia. 1 kb ladder DNA was used as a reference.

LEI0258 locus diversity analysis

The genotypic data were subjected to various within and among populations genetic diversity analyses. These included: calculation of the total number of alleles, allelic frequency and their distribution among the entire populations, polymorphic information content (PIC) for each population, Shannon's Information Index using the GenAlEx software package version 6.5 (Peakall and Smouse, 2012). Observed heterozygosity (*Ho*), expected (*He*) heterozygosity was estimated using the formula: $He = 1 - \sum (p^2_i + q^2_i)$ where p is the allelic frequency of the allele one at a given locus and q is the frequency of the alternate allele at the same locus (Ncube *et al*., 2014). The deviation of each population from Hardy-Weinberg Equilibrium (HWE) was also tested using GenAlEx software package. Number of homozygote and heterozygote genotypes were calculated using power marker analysis (Liu *et al*., 2002).

Sequence data management and analysis

In addition, high quality sequence reads with base call accuracy higher than 95% were assembled and resolved for conflicts using Qiagen's CLC work bench version 7. The resulting consensus sequences of chicken populations were aligned using ClustalW program integrated in the MEGA (Molecular Evolutionary Genetics Analysis) software version 7 (Kumar *et al*., 2016). For this, a reference homologous sequences of LEI0258 marker (accession no. DQ239495.1) with SNP position was downloaded from the National Center for Biotechnology Information (NCBI). The aim of blasting was to ascertain the sequenced alleles if they were novel to Ethiopian indigenous chicken populations or common to the globe. The haplotype tree was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-985.1747) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+G, parameter = 0.0500)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.

RESULTS

LEI0258 locus diversity from capillary electrophoresis based on sampling sites

We identified 29 LEI0258 alleles (100 genotypes; 64 heterozygotes and 36 homozygotes) in 24 populations from analysis of allelic variability by capillary electrophoresis. Allele sizes range from 197 to 569 bp. Size and frequency of the alleles are presented at Table 1. Six private alleles were detected in Surta (315,385), Ashuda (411), Meseret (185), Kumato (277) and Hadush Adu (465) populations although at 5%, 10%, 5% and 5% frequencies, respectively. Whereas 5 alleles (with their alleles in parentheses): 315 (8.2 %), 197 (12.9 %); 363 (8.2), 263 (8.1%) and 253 (7.5%), were the most frequent alleles across chicken populations. Only one allele (Allele 315) was common to all chicken populations, and 23 alleles were shared by at least by 2 chicken populations. Arabo chicken population has the highest effective number of alleles (11.76) and Hugub chicken populations the lowest (4.0). The power marker analysis also shows that the major allelic frequency and gene diversity are 16.31% and 91.9% across populations.

Population genetic diversity

Analysis of data from capillary electrophoresis indicated over all PIC at LEI0258 marker to be 0.98. The overall mean observed (Ho) among the entire population was 0.82., with He ranging from 0.5 in Shubi Gemo to 1 in Arabo, Gesses and Hadush Adi populations. The expected (He) heterozygosity was 0.85. An average of 2.06 Shannon index was obtained across populations. From the entire populations, only Kumato populations meet the assumption of Hardy Weinberg Equilibrium (HWE) (Table 2). The average inbreeding coefficients (F_{IS}) was 0.08.

Distribution of total genetic variation among chicken ecotypes

Results from AMOVA indicated 89% of the total genetic variation was due to differences within individual birds. Only 3% of the variation among studied chicken populations. Eight percent of the difference accounted due to difference among individuals (Figure 3).

Genetic distance and relationships

Table 3 and 4 summarizes the genetic distance (D_A) and gene differentiation (F_{ST}) indices among Ethiopian chicken populations, respectively. From, genetic distance calculation from capillary electrophoresis, showed the highest D_A (2.217) between Surta (Population from North West Ethiopia) and Hugub (Population from Eastern Ethiopia) chicken populations of Ethiopia. In other words, Hugub populations are genetically distant from most of the chick populations of Ethiopia. Whereas, Alfamidir chicken populations are the most genetically distinct with the majority of

the chicken populations (13 populations), followed by Metkilimat (12 populations and Mihiquan (11 populations) chicken populations. Improved Horro is the second most distance chicken population following Hugub chicken populations. A Hugub chicken population has the highest F_{ST} (0.12) with Batambie and ShubiGemo chicken populations). The lowest F_{ST} (0.01) was reported between Alfamidir and Batambie; Gijet and Tsion Teguaz; Metkilimat and Tsion Teguaz and Mihquan and Hadush Adi).

On the other hand, Figure 4 shows genetic relationships among chicken populations using PCA. The first two PC accounted for 30.4% of the total genetic variation. The first PC explained for 12.68% and the second PC explained an additional 17.72% of the variation. Both components didn't separate the 25 chicken populations into distinct clusters. However, 4 roughly admixed sub-clusters were formed with a few individual outliers.

Allelic sequence polymorphisms

Sequence information including repeat regions and flanking regions are available in https://blast.ncbi.nlm.nih.gov/Blast.cgi for a subset of homozygote LEI0258 genotypes. Based on the blastn information from NCBI, there are thirteen new alleles from the 21 alleles sequenced submitted and given accession numbers: MG495227, MG495229, MG495230, MG495231, MG495232, MG495233, MG495234, MG495235, MG495236, MG495237, MG495238, MG495239, MG495240, MG495241, MG495243, MG495244, MG495245, MG495246, MG495247, MG495248, and MG495249. Variable Number of Tandem Repeats (VNTR) R12 is observed 2 to 17 times but R13 (CTATGTCTTCTTT') only one.

The 23rd to 30th nucleotides downstream of the repeat region are "ATTTTGAG", whilst, 3 alleles sequences were found to have different repeats than respective reference sequences. 24 and 1 SNP substitutions were found at positions 39 and 46, respectively. 12 insertion SNPs and 2 deletions were noted on the upstream polymorphism positions of -30 to -29 positions. Besides, 2 nucleotide substitutions were reported at -61 upstream polymorphism, while, 3 substitutions at -28 position. Further polymorphisms were also observed in different positions of the repeat structure other than the positions considered here under. B10, B11.1, B13, B72 haplotypes were obtained from the allelic sequence. The invariable (monomorphic sites) and variable (polymorphic) sites found were 412 and 35, respectively. A total of 26 singleton sites and 5 parsimony informative sites were observed from the package of DNA sequence polymorphism while the total number of mutation sites and indel events were 33 and 17, respectively. The number of indel haplotypes and indel diversity were 5 and 0.00465. Haplotype diversity (Hd) was 0.82. The haplotype based phylogenic analysis using Neighbor Joining (NJ) showed that indigenous chicken populations are mainly clustered into two gene pools comprising different subpopulations as obtained from the structure analysis of allele sizes from

capillary electrophoresis (Figure 5). Genetic distance from subset sequencing showed distant alleles between Hugub and Surta chicken populations (Table 5).

Table 1. Allele frequencies of locus LEI0258 in Ethiopian indigenous chicken populations based on sites.

Allele/n	B	S	AM	GA	AD	ME	IH	HU	KE	GE	KI	TS	AR
N	8	9	10	10	10	25	30	10	10	10	10	10	10
185	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
197	0.31	0.17	0.00	0.15	0.05	0.08	0.20	0.00	0.10	0.35	0.15	0.00	0.15
209	0.00	0.00	0.05	0.00	0.05	0.04	0.00	0.10	0.00	0.00	0.00	0.00	0.05
221	0.06	0.00	0.05	0.00	0.25	0.10	0.08	0.05	0.00	0.00	0.00	0.00	0.00
245	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
253	0.06	0.00	0.10	0.05	0.00	0.04	0.05	0.00	0.05	0.10	0.10	0.20	0.05
263	0.00	0.00	0.15	0.05	0.05	0.06	0.15	0.25	0.00	0.15	0.05	0.00	0.15
277	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
289	0.06	0.00	0.05	0.10	0.00	0.00	0.00	0.00	0.00	0.10	0.25	0.05	0.05
300	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.10	0.05	0.00	0.10	0.05
302	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
312	0.00	0.17	0.00	0.10	0.15	0.10	0.17	0.00	0.05	0.05	0.00	0.25	0.05
315	0.31	0.17	0.20	0.30	0.15	0.38	0.07	0.10	0.10	0.05	0.10	0.20	0.05
325	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.05
327	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.05	0.00
340	0.00	0.00	0.00	0.00	0.25	0.00	0.28	0.05	0.20	0.00	0.05	0.00	0.05
351	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.10	0.05	0.05	0.00	0.00
363	0.06	0.22	0.25	0.05	0.00	0.06	0.00	0.00	0.20	0.05	0.10	0.10	0.10
375	0.13	0.00	0.10	0.20	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.05
385	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.05
397	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
411	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
426	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.05	0.00	0.00
450	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
460	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.05
465	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
472	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05
485	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.05	0.00
525	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
569	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 1. Continued ...

Allele /n	AS	DI	BG	SH	KU	LO	HA	MI	GI	ML	AL	NA	f
N	10	10	10	10	10	10	10	9	10	10	10	10	
185	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.001
197	0.10	0.10	0.15	0.05	0.05	0.10	0.15	0.06	0.10	0.20	0.25	0.20	0.129
209	0.00	0.00	0.00	0.05	0.00	0.05	0.05	0.00	0.00	0.00	0.00	0.00	0.018
221	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.028
245	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.005
253	0.05	0.10	0.05	0.10	0.00	0.10	0.05	0.11	0.20	0.15	0.15	0.00	0.075
263	0.05	0.10	0.00	0.00	0.10	0.05	0.15	0.06	0.05	0.10	0.05	0.25	0.081
277	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.002
289	0.15	0.15	0.00	0.00	0.05	0.10	0.05	0.06	0.10	0.05	0.10	0.00	0.059
300	0.00	0.00	0.15	0.35	0.00	0.05	0.10	0.06	0.00	0.00	0.00	0.00	0.042
302	0.00	0.00	0.05	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.008
312	0.10	0.15	0.10	0.15	0.00	0.00	0.00	0.17	0.15	0.05	0.05	0.10	0.084

315	0.25	0.20	0.15	0.05	0.20	0.15	0.15	0.11	0.25	0.15	0.25	0.05	0.165
325	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.006
327	0.00	0.15	0.00	0.00	0.00	0.10	0.00	0.06	0.10	0.00	0.00	0.00	0.024
340	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.35	0.051
351	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.030
363	0.05	0.05	0.05	0.10	0.20	0.10	0.10	0.11	0.00	0.05	0.05	0.00	0.082
375	0.00	0.00	0.10	0.00	0.15	0.05	0.05	0.00	0.00	0.10	0.10	0.05	0.044
385	0.10	0.00	0.00	0.05	0.00	0.10	0.00	0.06	0.00	0.00	0.00	0.00	0.015
397	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.05	0.00	0.00	0.005
411	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.004
426	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.008
450	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.004
460	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.010
465	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.002
472	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.004
485	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.005
525	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.001
569	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.008

BA = Batambe; SU = Surta; AM = Amesha Shinkuri; GA = Gafera; AD = 025-Adane; ME = Meseret; IH = Improved Horro; HU = Hugub; KE = Kefis; GE = Gesses; KI = Kido; TT = Tsion Teguaz; AR = Arabo; AS = Ashuda; DI = Dikuli; BG = Bekele Girisa; SG = Shumbi Gemo; KU = Kumato; LO = Loya; HA = Hadushi Adi; MI = Mihiquan; GI = Gijet; ML = Metkilimat; AL = Alifa Midir; NA = Negasi Amba; f = Average frequency.

Table 2. Diversity indices of LEI0258 locus in Ethiopian indigenous chicken populations based on sampling sites.

Pop	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**uHe**	**F**	**HWE (p-value)**	**PAL**
Batambe	8	7.0	4.4	1.68	0.75	0.77	0.83	0.030	0.21	
Surta	9	7.0	6.2	1.88	0.67	0.84	0.89	0.206	0.20	315, 385
Amesha_Shinkuri	10	9.0	6.5	2.01	1.00	0.85	0.89	-0.183	0.89	
Gafera	10	8.0	5.6	1.878	0.80	0.82	0.86	0.024	0.2	
025_Adane	10	8.0	5.6	1.86	0.70	0.82	0.86	0.146	0.08	
Meseret	10	12.0	7.7	2.26	1.00	0.87	0.92	-0.149	0.68	185
Hugub	10	7.0	4.00	1.62	0.70	0.75	0.79	0.07	0.09	
Kefis	10	10.0	7.69	2.16	0.70	0.87	0.92	0.20	0.32	
Gesses	10	10.0	5.56	2.01	1.00	0.82	0.86	-0.22	0.93	
Kido	10	10.0	7.4	2.15	0.80	0.87	0.91	0.06	0.15	
Tsion_Teguaz	10	8.0	5.9	1.90	0.80	0.83	0.87	0.04	0.35	
Arabo	10	15.0	11.7	2.60	1.00	0.92	0.96	-0.09	0.71	
Ashuda	10	10.0	7.41	2.15	0.80	0.87	0.91	0.06	0.42	411
Dikuli	10	8.0	7.14	2.02	0.8	0.86	0.91	0.07	0.31	
Bekele_Girisa	10	11.0	9.1	2.29	0.9	0.89	0.94	-0.01	0.47	
Shubi_Gemo	10	9.0	5.41	1.942	0.5	0.82	0.86	0.387	0.24	
Kumato	10	9.0	7.14	2.068	0.8	0.86	0.91	0.07	0.02*	277
Loya	10	12.0	10.5	2.415	0.9	0.91	0.95	0.01	0.57	
Hadush_Adi	10	12.0	9.52	2.36	1.0	0.90	0.94	-0.12	0.49	263
Mihiquan	9	12.0	10.1	2.4	0.89	0.9	0.95	0.01	0.33	
Gijet	10	8.0	6.25	1.94	0.70	0.84	0.88	0.17	0.39	
Metkilimat	10	11.0	8.33	2.25	0.90	0.88	0.93	-0.02	0.37	
Alfa_Midir	10	8.0	5.71	1.89	0.80	0.83	0.87	0.03	0.23	
Negassi_Amba	10	6.0	4.17	1.57	0.80	0.76	0.80	-0.05	0.83	
Mean		9.46	7.04	2.06	0.82	0.85	0.89	0.03		
SE		0.44	0.42	0.05	0.03	0.01	0.01	0.03		

PAL = Private allele; *He* = Unbiased Expected Heterozygosity/ Nei's Gene diversity = (2N / (2N-1)) * He; I = Shannon's Information Index = -1* Sum (pi * Ln (pi)); *significantly deviate from HWE ($P < 0.05$); AR=Allelic Richness

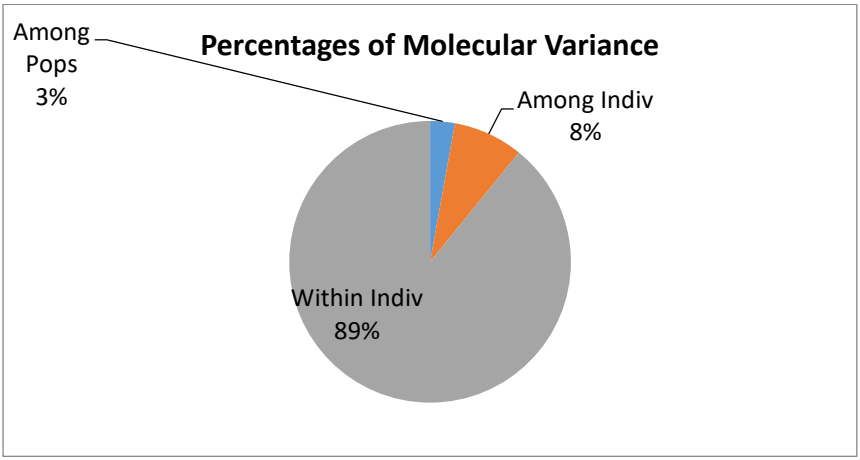

Figure 3. Results of Analysis of Molecular Variance across sampling sites

Table 3. Nei unbiased genetic distance between Ethiopian chicken populations.

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
0.000												
0.167	0.000											
0.299	0.221	0.000										
0.000	0.182	0.112	0.000									
0.683	0.749	0.851	0.670	0.000								
0.075	0.246	0.185	0.022	0.356	0.000							
0.634	0.645	1.116	0.690	0.058	0.782	0.000						
1.720	2.217	0.853	1.385	1.040	1.108	1.109	0.000					
0.441	0.116	0.187	0.541	0.326	0.602	0.241	0.797	0.000				
0.096	0.297	0.677	0.339	1.230	0.800	0.336	0.999	0.464	0.000			
0.164	0.285	0.282	0.207	1.082	0.664	0.668	1.115	0.289	0.006	0.000		
0.567	0.169	0.245	0.225	0.667	0.226	0.815	2.098	0.352	0.827	0.454	0.000	
0.000	0.001	0.000	0.000	0.402	0.320	0.000	0.517	0.000	0.000	0.000	0.311	0.000
0.001	0.147	0.187	0.000	0.612	0.000	0.668	1.297	0.503	0.294	0.000	0.087	0.000
0.135	0.083	0.186	0.000	0.600	0.117	0.484	1.144	0.531	0.181	0.000	0.000	0.000
0.000	0.074	0.251	0.000	0.726	0.156	0.562	1.192	0.041	0.119	0.408	0.050	0.000
1.282	0.516	0.657	1.077	1.382	1.106	1.279	2.851	0.345	0.843	1.244	0.175	0.274
0.081	0.175	0.000	0.000	0.561	0.126	1.002	0.990	0.244	0.683	0.348	0.560	0.000
0.000	0.044	0.000	0.000	0.907	0.070	0.749	1.123	0.138	0.038	0.000	0.031	0.000
0.000	0.105	0.000	0.000	0.528	0.090	0.227	0.610	0.000	0.000	0.000	0.314	0.000
0.253	0.000	0.000	0.017	0.516	0.104	0.384	0.346	0.000	0.091	0.000	0.000	0.000
0.105	0.244	0.316	0.000	0.623	0.068	0.574	1.426	0.632	0.308	0.043	0.000	0.127
0.000	0.151	0.060	0.000	0.749	0.146	0.327	1.075	0.359	0.000	0.000	0.187	0.000

0.000	0.162	0.187	0.000	0.805	0.102	0.510	1.490	0.408	0.000	0.000	0.229	0.000
0.796	0.938	1.041	0.758	0.222	1.098	0.000	0.881	0.261	0.364	0.722	1.513	0.000

Table 3. Continued ...

14	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**Pop**
												Batambe
												Surta
												Amesha_Shinkuri
												Gafera
												025_Adane
												Enderta
												Improved_Horro
												Hugub
												Kefis
												Gesses
												Kido
												Tsion_Teguaz
												Arabo
0.000												Ashuda
0.000	0.000											Dikuli
0.108	0.136	0.000										Bekele_Girisa
0.865	0.793	0.024	0.000									Shubi_Gemo
0.172	0.268	0.136	1.273	0.000								Kumato
0.000	0.000	0.000	0.355	0.000	0.000							Loya
0.000	0.000	0.000	0.295	0.000	0.000	0.000						Hadush_Adu

0.000	0.000	0.000	0.036	0.170	0.000	0.000	0.000					Mihiquan
0.000	0.000	0.098	0.760	0.477	0.000	0.088	0.000	0.000				Gijet
0.000	0.000	0.000	0.822	0.043	0.000	0.000	0.000	0.000	0.000			Metkilimat
0.000	0.000	0.000	0.957	0.136	0.000	0.000	0.000	0.000	0.000	0.000		Alfa_Midir
0.889	0.676	0.761	1.813	0.964	0.829	0.125	0.706	0.931	0.386	0.646	0.000	Negassi_Amba

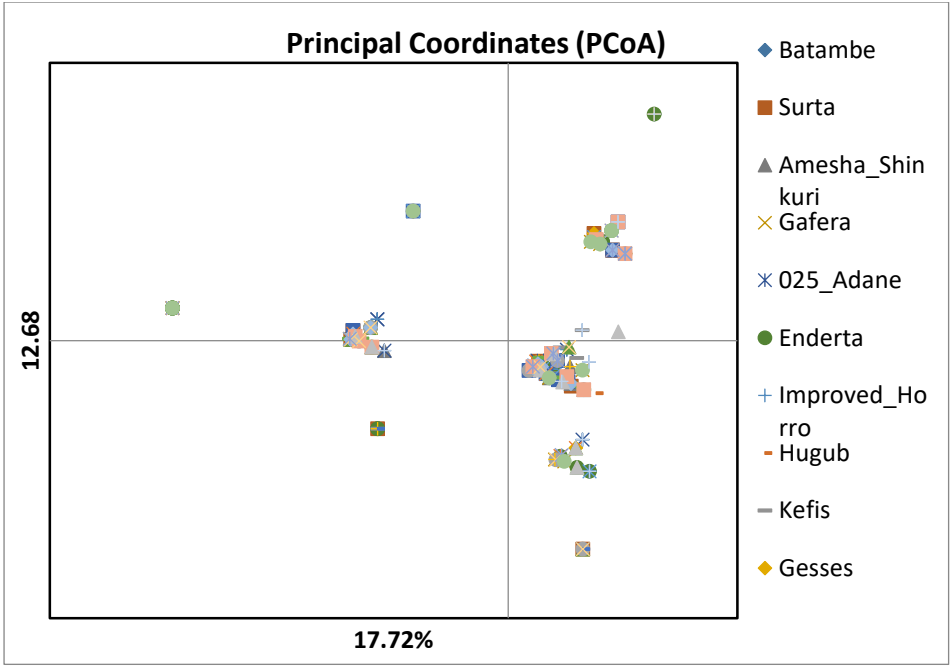

Figure 4. Principal component graph of the first 2 principal components from 25 chicken populations

Table 4. Pairwise Population F_{ST} values across sampling sites.

PO	BA	SU	AS	GA	AD	EN	HU	KE	GE	KI	TT	AR
BA	0											
SU	0.04	0										
ASH	0.05	0.04	0									
GA	0.02	0.04	0.03	0								
AD	0.07	0.06	0.06	0.06	0							
EN	0.05	0.03	0.02	0.03	0.04	0						
HU	0.12	0.11	0.08	0.10	0.09	0.08	0					
KE	0.06	0.03	0.04	0.05	0.04	0.04	0.07	0				
GE	0.04	0.05	0.06	0.05	0.08	0.06	0.09	0.05	0			
KI	0.04	0.04	0.04	0.04	0.07	0.05	0.08	0.04	0.03	0		
TT	0.07	0.04	0.04	0.04	0.06	0.02	0.11	0.04	0.07	0.05	0	
AR	0.04	0.03	0.02	0.03	0.05	0.03	0.06	0.02	0.02	0.02	0.04	0
AS	0.03	0.03	0.04	0.02	0.05	0.02	0.09	0.04	0.04	0.03	0.03	0.03
DI	0.04	0.03	0.04	0.02	0.05	0.03	0.09	0.05	0.04	0.02	0.02	0.03
BG	0.03	0.03	0.04	0.02	0.06	0.03	0.08	0.03	0.04	0.04	0.03	0.02
SG	0.09	0.06	0.06	0.08	0.08	0.05	0.12	0.05	0.07	0.07	0.04	0.04
KU	0.04	0.04	0.01	0.02	0.05	0.03	0.08	0.04	0.06	0.04	0.05	0.03
LO	0.03	0.03	0.02	0.03	0.06	0.02	0.08	0.03	0.03	0.02	0.03	0.01
HA	0.03	0.03	0.02	0.03	0.05	0.03	0.07	0.02	0.02	0.02	0.04	0.01
MI	0.05	0.03	0.03	0.03	0.05	0.02	0.06	0.02	0.04	0.03	0.01	0.02
GI	0.04	0.04	0.04	0.02	0.06	0.03	0.10	0.05	0.04	0.03	0.01	0.04
ME	0.02	0.04	0.03	0.02	0.06	0.03	0.08	0.04	0.02	0.03	0.04	0.01
AL	0.01	0.04	0.04	0.01	0.07	0.04	0.10	0.05	0.02	0.03	0.04	0.03
NA	0.09	0.08	0.08	0.08	0.05	0.08	0.10	0.05	0.06	0.07	0.10	0.04

Table 4. Continued …

PO	AS	DI	BG	SG	KU	LO	HA	MI	GI	ME	AL	NA
BA												
SU												
ASH												
GA												
AD												
EN												
HU												
KE												
GE												
KI												
TT												
AR												
AS	0											
DI	0.02	0										
BG	0.03	0.03	0									
SG	0.06	0.06	0.03	0								
KU	0.03	0.04	0.03	0.07	0							
LO	0.02	0.02	0.02	0.05	0.02	0						
HA	0.02	0.03	0.02	0.04	0.02	0.01	0					

MI	0.02	0.02	0.02	0.04	0.03	0.02	0.02	0				
GI	0.02	0.01	0.03	0.06	0.05	0.02	0.03	0.02	0			
ME	0.02	0.02	0.02	0.06	0.03	0.02	0.01	0.02	0.02	0		
AL	0.02	0.02	0.02	0.07	0.03	0.02	0.02	0.03	0.02	0.01	0	
NA	0.08	0.07	0.07	0.11	0.08	0.07	0.04	0.07	0.08	0.06	0.07	0

BA = Batambie; SU = Surta; ASH = Amesha Shinkuri; GA = Gafera; AD = 025-Adane; ME= Meseret; HU= Hugub; KE = Kefis; GE = Gesses; KI = Kido; TT = Tsion Teguaz; AR = Arabo; AS = Ashuda; DI = Dikuli; BG = Bekele Girisa; SG = Shumbi Gemo; KU = Kumato; LO = Loya; HA = Hadushi Adi; MI = Mihiquan; GI = Gijet; ME = Metkilimat; AL = Alifa Midir; NA = Negasi Amba.

Table 5. Genetic distance from subset sequencing showing identical and distant alleles.

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
0	23.8	49.2	26.4	48.7	48.7	48.7	48.7	48.7	48.7	48.7	25.9	25.9
	0	48.4	21.7	51.2	51.2	51.2	51.2	51.2	51.2	51.2	22.6	22.6
		0	46.2	50.4	50.4	49.6	50.0	50.0	50.4	50.4	48.3	48.3
			0	53.6	53.6	51.6	51.6	54.0	54.0	54.0	20.8	20.8
				0	0	15.2	14.8	17.3	16.6	16.6	51.6	51.6
					0	15.2	14.8	17.3	16.6	16.6	51.6	51.6
						0	0.3	14.9	14.6	14.6	52.9	52.9
							0	15.3	14.2	14.2	52.9	52.9
								0	1.0	1.0	54.1	54.1
									0	0	54.1	54.1
										0.0	54.1	0
											0	0
												0

14	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**Chickens**
25.9	25.9	26.9	25.9	48.7	48.7	48.7	51.3	Batanbe_4H 1
22.6	22.6	23.5	22.6	51.2	51.2	51.2	51.6	Meseret_156b 3
48.3	48.3	48.3	48.3	50.4	50.4	50.4	60.2	Hugub_H2 4
21.2	21.2	22.0	21.2	54.4	54.4	54.4	54.4	Tsion_9C 5
53.4	51.9	53.7	53.7	19.4	19.4	19.4	51.6	Ashuda_1C 6
53.4	51.9	53.7	53.7	19.4	19.4	19.4	51.6	Kefis_12C 7
52.5	53.9	52.5	54.2	19.0	19.0	19.0	51.9	BekeleGirisa_8
52.5	53.9	52.5	54.2	18.6	18.6	18.6	52.2	ShubiGemo_1H
53.1	53.1	54.7	53.4	18.6	18.6	18.6	53.1	Ashuda_10H 10
53.1	52.8	54.4	53.1	17.9	17.9	17.9	53.1	Dikuli_4H 11
53.1	52.8	54.4	53.1	17.9	17.9	17.9	53.1	Gijet_49H 12
13.6	14.9	17.2	17.8	55.7	55.7	55.7	51.8	Gafera_8C 13
13.6	14.9	17.2	17.8	55.7	55.7	55.7	51.8	Ashuda_9C 14
0	13.1	15.3	15.9	57.0	56.7	56.7	52.0	Dikuli_7H 15
	0	12.9	13.8	55.9	56.2	56.2	53.5	NgasiAmba_4H
		0	12.8	58.0	58.0	58.0	55.1	Hugub_H9 18
			0	57.7	57.7	57.7	54.9	Adane_9C 19
				0	0.3	0.3	55.4	BekeleGirisa_H
					0	0	55.2	Kumato_2C 21
						0	55.2	Kumato_5H 22
							0	Surta_7H 23

Table 6. Overall polymorphisms identified in the LEI0258 alleles in indigenous chickens of Ethiopia.

[a]Chicken	NCBI Acc. No.		Fragment length (bp, by genotyping)	Consensus size (bp, by sequencing)			Upstream	R13		R12	Downstream		Acc. No.		Haplotype
				Δ	TT	G	TT			C	ATTTGAG	Δ	T	T	
				-61	-30 to -29	-28	-19-18			5	23-30	33	39	46	
Batambe_4H	MG495227	197	193	∘	∘	∘	∘	1	2	∘	Δ	∘	Δ	Δ	DQ239495
Surta_7H*	MG495249	315/426	486	∘	△	△	∘	1	13	∘	∘	∘	Δ	∘	DQ239562
Hugub_H2*	MG495230	351/460	236	∘	△	∘	∘	1	4	∘	∘	∘	Δ	∘	KF535086
Hugub_H9*	MG495244	351	345	∘	∘	∘	∘	1	15	∘	∘	∘	Δ	∘	DQ239508
Gafera_8C*	MG495239	315	309	∘	∘	∘	∘	1	12	∘	∘	∘	Δ	∘	KF534941
Tsion_9C*	MG495231	315/327	250	∘	∘	∘	∘	1	7	∘	∘	∘	Δ	∘	KF534930
Adane_9C*	MG495245	312/340	357	∘	∘	∘	∘	1	16	∘	∘	∘	Δ	∘	DQ239506
Ngasiamba_4H	MG495243	340	333	∘	∘	∘	∘	1	14	∘	∘	∘	Δ	∘	KF534946
Ashuda_1C	MG495232	289	283	∘	△	△	△	1	10	∘	∘	∘	Δ	∘	KF535091

Ashuda_9C*	MG495240	315	309	∘	∘	∘	∘	1	12	∘	∘	∘	Δ	∘	DQ239494
Ashuda_10H*	MG495236	289	295	∘	△	∘	∘	1	11	∘	∘	∘	Δ	∘	DQ239550
Dikuli_4H*	MG495237	315/411	307	∘	△	△	△	1	12	∘	∘	∘	Δ	∘	DQ239550
Kefis_12C	MG495233	289	283	∘	△	△	△	1	10	∘	∘	∘	Δ	∘	KF535091
Dikuli_7H	MG495241	321	321	∘	∘	∘	∘	1	13	∘	∘	∘	Δ	∘	KF534945
Bekelegirisa_1H*	MG495246	300/302	379	∘	△	△	△	1	18	∘	∘	∘	Δ	∘	KF535100
Bekelegirisa_8H*	MG495234	312/315	295	∘	△	△	△	1	11	∘	∘	∘	Δ	∘	KF534937
Shubigemo_1H*	MG495235	300	295	∘	△	△	△	1	11	∘	∘	∘	Δ	∘	DQ239496
Kumato_2C	MG495248	197/460	357	∘	△	△	△	1	16	∘	∘	∘	Δ	∘	KF535100
Kumato_5H	MG495248	302	379	∘	△	△	△	1	18	∘	∘	∘	Δ	∘	KF535100
Meseret_156b	MG495229	312/315	333	∘	∘	∘	∘	1	4	∘	Δ	∘	Δ	∘	KF534926
Gijet_49H*	MG495238	253	295	∘	△	∘	△	1	12	∘	∘	∘	Δ	∘	DQ239550

[a]The codes of chicken populations; [b]Δ Defined deletion compared with the reference sequence. ◦Identical to the reference sequence; * unique alleles

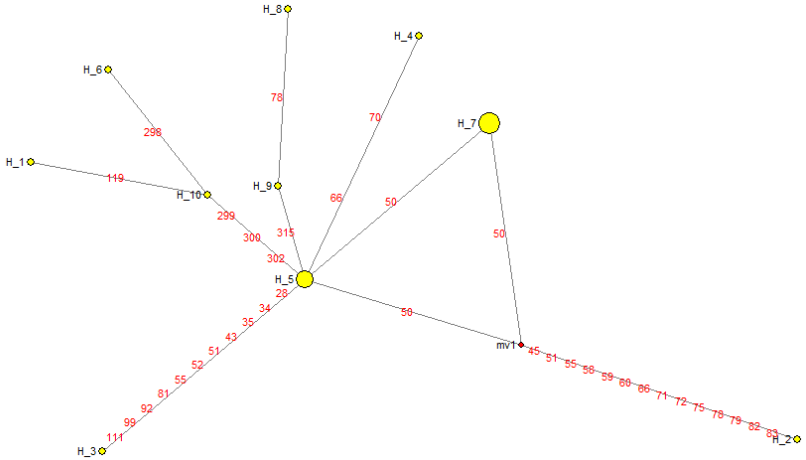

Figure 5. Haplotypes relationship tree of alleles by the Maximum Likelihood method

Table 7. NCBI accession numbers of Raw sequences of indigenous chicken of Ethiopia

S/No	Chicken sample identity	New accession numbers
1	Batambe_4H	MG495227
2	Meseret_156b	MG495229
3	Hugub_H2	MG495230
4	Tsion_9C	MG495231
5	Ashuda_1C	MG495232
6	Kefis_12C	MG495233
7	BekeleGirisa_8H	MG495234
8	ShubiGemo_1H	MG495235
9	Ashuda_10H	MG495236
10	Dikuli_4H	MG495237
11	Gijet_49H	MG495238
12	Gafera_8C	MG495239
13	Ashuda_9C	MG495240
14	Dikuli_7H	MG495241
15	NegasiAmba_4H	MG495243
16	Hugub_H9	MG495244
17	Adane_9C	MG495245
18	BekeleGirisa_H1	MG495246
19	Kumato_2C	MG495247
20	Kumato_5H	MG495248
21	Surta_7H	MG495249

DISCUSSION

This study aimed at assessing the allelic and genetic diversity as well as relationship of 25 chicken populations in Ethiopia using MHC-linked LEI0258 Marker so as to ascertain the importance of these ecotypes for genetic improvement through selection and conservation. Genotyping by both fragment and Sanger sequencing analysis were employed. In this study, we report polymorphism at the MHC-B VNTR marker, LEI0258, in indigenous chicken village chicken populations from Ethiopia. From Capillary electrophoresis, a total of 29 alleles were scored from 25 chicken populations with size ranging 185 to 569 bp with alleles frequencies ranging from 2.5% to 38% across populations. Out of the 29 alleles, only 1 allele was observed in all 25 chicken populations, whereas, 6 private alleles were observed in only one population each. The high allelic variability at the marker may

be a direct consequence of the diversity of disease challenges facing Ethiopian chicken within and across different agro-ecologies, with polymorphism at the locus maintained by balancing selection with high LEI0258 diversity increasing the diversity of antigens being presented to T-cells (Chazara *et al*., 2013). The number of alleles reported here is higher than the numbers reported by different authors in previous works on indigenous chicken populations (Han *et al*., 2013; Nikbakht *et al*., 2013). From the 29 alleles reported here, 22 are novel alleles that were found in only one population each. The maximum allele size reported in this study is larger than the one characterized in other studies (e.g., Han *et al*. 2013). Overall, 50% of the alleles in this study are only found in only two or three of the populations out of the 24 considered. Except for alleles, 197, 312, 315 and 351 bp, the remaining of the alleles occurred at lower frequency (< 0.20). The presence of non-frequent alleles across chicken populations suggested distance relationship in all study populations. The low frequency abundance of LEI0258 alleles might be attributed to a new mutation arising in a population and therefore available only in few individuals. It might also be due to its susceptibility to disease and other selection pressures resulting unfit to survive the production challenge the chicken is facing in the variable environments where they were sampled. High frequency of allele 315 at Batambe population could imply a fitness or survival advantage to the individual carrying it resulting in it being selected for and occurring at higher frequencies (31%). This however needs to be further studied in absence of any information regarding the possible association between disease resistance/susceptibility and the allele. The minimum and maximum numbers of alleles per population were 6 and 15, respectively. The large numbers of and big ranges of allele sizes at LEI028 marker implies high allelic polymorphisms across studied chicken populations. Variations in allele numbers among populations might be an indication of differences of chicken populations, their origins, dispersion, production environments, and level of interactions within and between populations.

We did not identify here those alleles which previously have been shown to be positive correlation with NDV (206 bp) and body weight (307 bp) (Fulton *et al*., 2006; Lwelamira *et al*. ; Fulton *et al*., 2016a. For the later, it may not be surprising considering the small size of Ethiopian village chicken compared to their commercial counterparts; while the former suggests that allele (206 bp) may not be of relevance to NDV resistance/susceptibility in Ethiopian village chicken. Several LEI0258 alleles were shared among the predetermined populations implying that they have been subjected to either directional selection or due to recombination effect (Nikbakht *et al*., 2013; Salomonsen *et al*., 2014). Alleles 205 and 307 bp, reported in Tanzanian chicken to associate with Newcastle disease antibody response and body weight, respectively, were not reported in Ethiopian chicken in this study (Lwelamira *et al*., 2008). The power marker analysis also showed that the minor allelic frequency of the entire population was 17%.

From Sanger sequencing analysis, 2 levels of polymorphisms were considered, i.e., two repeat regions or motifs: R13 (CTATGTCTTCTTT) and R12 (CTTTCCTTCTTT) and indels and SNP along both flanking regions. A subset of allele sequencing result ascertained the presence of single nucleotide polymorphisms (SNPs) in LEI0258. The two main VNTR were the R13 and R12. R13 with a 13 bp repeat unit, "CTATGTCTTCTTT' was found with frequency of only once similar to Wang *et al*. (2014) and inconsistent with other studies with more frequencies (Chazara *et al*., 2013; Nikbakht *et al*., 2013). The 23 to 30 position downstream of the repeat region was sequenced as "ATTTTGAG". They agree with the sequences reported before (Fulton *et al*., 2016a; Han *et al*., 2013). Twenty-four and one SNP substitutions were found at positions 39 and 46, respectively. The number of R12 motifs (CTTTCCTTCTTT) in the individual sequences ranged from 2 to 18, whilst, only one R13 motif was found. Twelve insertion SNPs and 2 deletions were noted on the upstream polymorphism positions of -30 to -29 positions. Besides, 2 nucleotide substitutions were reported at -61 upstream polymorphism, while, 3 substitutions at -28 positions. 6 allele sequences were found to have different repeats than respective reference sequences (ATTTTGAG). Results of allele size from both fragment length and consensus sequences did not exactly much which might be because of the difference in environmental factors, technological approaches and precisions. The size deviation ranged from 1 to 115 unlike what was reported by Han *et al*. (2013) who found size differences of 1 to 69 ranges. The comparison between the fragment sizes and consensus sizes (bp) across population consistently showed higher values for the later except for the Dara population. This result was not consistent with the works of Fulton (2016a) and Han *et al*. (2013). Further polymorphisms were also observed in different positions of the repeat structure other than the positions considered here under. Only few haplotypes (B10, B11.1, B13, B72), were found when compared with the haplotypes reported by Chazara *et al.* (2013). In contrast, none of these haplotypes were reported in the report of Wang *et al*. (2014) for Chinese chicken and Ngeno *et al*. (2015) for Kenyan indigenous chicken. The haplotypes in this study are not in concordance with the haplotypes reported by Fulton *et al.* (2016a) in heritage breeds of chickens in Canada and the United States. Considering the haplotypes identified in our study, there is no any known history of European sources for indigenous chicken populations of Ethiopia.

The SNP based phylogenic analysis using Neighbor Joining (NJ) showed that indigenous chicken populations are mainly clustered into two gene pools comprising different subpopulations as obtained from the structure analysis of allele sizes from capillary electrophoresis. From phylogenetic analysis clear separation of ecotypes were not noted indicating genetic admixtures between populations.

Analysis of data from Capillary electrophoresis indicated over all PIC at LEI0258 marker to be 0.98. The overall mean observed heterozygosity (Ho) among the entire population

was 0.82., with He ranging from 0.5 in Shubi Gemo to 1 in Arabo, Gesses and Hadush Adi populations. The expected (He) heterozygosity was 0.85. The mean observed heterozygosity (82.1%) found in this study are far more than the expected level (50%) indicating higher genetic variation in indigenous chicken populations of Ethiopia. In other words, a heterozygous population has a better degree of resisting/tolerating multiple disease infections that challenge chicken populations. From the entire populations, Kumato populations are found to be significantly different from the test of the Hardy Weinberg Equilibrium (HWE). The possible factors that cause this deviation from HWE might be possible introgression with exotic chicken as they are very close to the exotic poultry multiplication center. The other possible reasons might be due to a continuous exposure to disease and parasite challenges (viral/ bacterial infections) that indigenous chicken are exposed to the occurrence of natural selection at MHC locus to combat these challenges. Besides, our sampling might have target related birds.

One of the main objectives of this study was to determine the genetic relationship of 25 Ethiopian chicken populations in order to evaluate if they can be considered as distinct populations for further genetic improvement and conservation. Coefficients of genetic differentiation (F_{ST}), standard genetic distance (DA), and PCA were employed to evaluate genetic relationships. Genetic distance calculation from capillary electrophoresis, showed the highest D_A (2.217) between Surta (Population from North West Ethiopia) and Hugub (Population from Eastern Ethiopia) chicken populations of Ethiopia. This might be due to the fact that the difference in entry of introduction of chicken to Ethiopia. In other words, Hugub populations are genetically distant from most of the chick populations of Ethiopia. Whereas, Alfamidir chicken populations are the most genetically distinct with the majority of the chicken populations (13 populations), followed by Metkilimat (12 populations and Mihiquan (11 populations) chicken populations. A Hugub chicken population has the highest F_{ST} (0.12) with Batambie and ShubiGemo chicken populations). The lowest F_{ST} (0.01) was reported between Alfamidir and Batambie; Gijet and Tsion Teguaz; Metkilimat and Tsion Teguaz and Mihquan and Hadush Adi). The low genetic differentiation and distance between indigenous chicken populations of Ethiopia are attributed to high gene flows, admixture, and interbreeding in the study area as well as sharing a common ancestry among the populations. Improved Horro is the second most distance chicken population following Hugub chicken populations. The results from PCA also ascertained the close relationships among the chicken populations. This finding further evidences that the chicken populations are highly admixed or share a common ancestry and that their differentiation might be a recent one. The current result concurred with the findings of Mwambene *et al*. (2019).

CONCLUSION

Very high diversity was found in Ethiopian indigenous chicken populations at LEI0258, this diversity is observed within all populations. Our results support the importance of MHC diversity in response to the disease challenges faced by smallholder poultry production in Ethiopia. Breeding improvement programs will need to maximize this diversity through balancing selection that maintains polymorphisms and increases within population diversity. This very high diversity report for Ethiopian indigenous chicken populations on LEI0258 locus will provide a framework for the existing and future chicken breed improvement interventions. Besides, we can infer that the genotyping of the VNTR marker LEI0258 is a suitable method for MHC typing of indigenous Ethiopian chicken populations considering the high level of polymorphism observed at the locus within and across indigenous populations. Polymorphisms from the sequencing result, also support the genome diversity of indigenous Ethiopian village chicken populations. As a way forward, studying the relationship of these polymorphisms and the disease resistance/susceptibility haplotypes in Ethiopian chicken populations should be undertaken.

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