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Research Article
Genetic Diversity and Population Structure of Indigenous Chicken Ecotypes (*Gallus gallus domesticus*) in Ethiopia using LEI0258 Microsatellite

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Abstract

Background and Objective: The indigenous chickens (*Gallus gallus domesticus*) are widely distributed all part of Ethiopia and remain as the main animal protein source for humans. Even though few marker-based molecular characterizations have been conducted on them, none of them used the LEI0258 microsatellite. Therefore, this research project was initiated to see the polymorphism of the LEI0258 microsatellite marker in the Ethiopian indigenous chickens and its potential to study indigenous chicken ecotypes. **Materials and Methods:** 2-5 mL of the blood sample was collected from 25-30 chicken per sample site. Then, total genomic DNA was extracted using a Qiagen DNA extraction kit followed by PCR amplification and Sanger sequencing. **Results:** Thirty-seven forms of LEI0258 were identified and the marker has been found as highly polymorphic with polymorphic information content (PIC) of 0.9288. The marker was employed to analyze the pairwise genetic distance between ecotypes. As a result, the lowest genetic distance was observed between *Gema Gemmedaa* and *Ahun Tegegn* ecotypes (0.4950). The highest genetic distance was observed between *Sarbo* and *Gelego* ecotypes (0.8324). The nine chicken ecotypes sampled in a different part of the country grouped into three major clusters. The naked neck chicken ecotype was isolated from the others and forms a separate cluster. **Conclusion:** The majority of LEI0258 microsatellite forms are found in at least in two ecotypes, although there are a few alleles unique to a particular ecotype.

Key words: LEI0258 microsatellite, indigenous chicken, PIC, genetic diversity, population structure, Ethiopia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ethiopia has 60.5 million chicken as at 2016/17[1], of that, 94.31% are indigenous. Indigenous chickens are mainly managed by women and children under a free-range system[2-4]. They are important sources of protein and income for rural-poor and unemployed youth. Indigenous chicken is dispersed across different parts of Ethiopia[1]. Such widespread distribution signifies their adaptive potential to multiple local environmental conditions, diseases and other stresses[5].

It is believed that missionaries imported chicken to Ethiopia for the first time, nevertheless, there is no clear information when and how they were imported[5]. Since their importation, there is no significant improvement on chicken productivity even though there have been some crossbreeding attempts, albeit inconsistent and indiscriminate crossbreeding. Thus, Ethiopian indigenous chickens still resemble in body size and productivity their ancestors (red jungle fowl). Previous studies revealed that indigenous chickens in Ethiopia are varied in terms of morphology, feather distribution and plumage color[5-7]. Others differ concerning comb type, shank and skin color, feather pattern and body shape. Based on feather distribution, feathered, naked neck and frizzle ecotypes are common in various agro-ecological zones of the country[6]. Blocky, wedge and triangular are the dominant kinds of body shape in Ethiopian indigenous chicken ecotypes[7]. These kinds of variations could be attributed to co-evolution because of environmental change.

Only limited comprehensive phenotypic and molecular characterization studies have been conducted to identify and characterize the Ethiopian indigenous chicken ecotypes. The molecular characterization is predominantly conducted using microsatellite markers. Previous studies have been done by Tadelle[6], Halima[5] and Dana *et al*.,[7], however, no one has utilized the LEI0258 microsatellite to characterize the Ethiopian indigenous chickens. LEI0258 microsatellite has been globally proven a useful method for the study of village chicken diversity. This marker is increasingly being used in research and for planning cross-breeding and it has been found a successful tool for controlling the animals' MHC genotypes[8-10]. Since the polymorphism of LEI0258 microsatellite in Ethiopian indigenous chicken has not been studied and the marker not yet tested to characterize indigenous chicken found in Ethiopia, this research study was initiated.

MATERIALS AND METHODS

Description of the study area and population: Ethiopia is located in 8°00 N latitude and 38°00 E longitudes and has various agro-ecological zones that range from dry hot to alpine. The altitude ranges from -125-4200 m sea level as shown in Table1. In 2017, the total chicken population was 60.5 million chicken[11], of that, 94.31% are indigenous.

The study ecotypes named following the sample site include *Gelego*, *Ahun Tegegn*, *Sekella*, *Bakelit*, *Tula*, *Gumaidea*, *Gema Gemmedaa*, Sabro and *Dubie*. *Gelego* ecotype is the naked neck ("*Angete Melata*") ecotype, which is typically adapted to the hot climatic condition of the Northwestern part of Ethiopia. The body and behavioral characteristic of this ecotype are defeather at neck and chest (Fig. 1d) and it is highly aggressive[12]. *Ahun Tegegn* is crest headed ("*Gutena*") and small-sized ecotypes. It is common in the Northwestern highland part of the country. *Sekela* ecotype also known as "*Solola*" by the local community is characterized by long shank length and heavy body weight[6]. *Bakelit* ecotype is densely feathered and is common in the central highland part of the country. *Sarbo* and *Gemma Gemmedda* chicken ecotypes are largely distributed in the Southwestern part of Ethiopia and are characterized by a normal feather morphology and distribution. *Tula* and *Gumaidea* ecotypes have silky feathers and are found in the area lying within the rift valley. *Dubie* is a normal feather ecotype as is found in the Northwestern midland part of Ethiopia. *Gugut* ecotype is muffed and found around Sanja in the Northwestern part of the county[12]; they are few in numbers and for that reason were not included in the study.

Table 1: The traditional classification of the Ethiopian agro-ecological zones based on altitude, rainfall and temperature

Zone	Altitude (m)	Mean rainfall (mm)	Temperature (°C)
Bereha (dry-hot)	500-1,500	<900	>22
Weinadega (dry-warm)	1,500-2,500	<900	18-20
Erteb Kola (sub-moist warm)	500-1,500	900-1,000	18-24
Weinadega (sub-moist cool)	1,500-2,500	900-1,000	18-20
Erteb Weinadega (moist-cool)	1,500-2,500	>1,000	18-20
Dega (cold)	2,500-3,500	900-1,000	14-18
Erteb dega (moist cold)	2,500-3,500	>1,000	10-14
Wurch (very cold or alpine)	>3,500	>1,000	<10

Fig. 1(a-f): Some of the indigenous chicken ecotypes found in Ethiopia (a) *Ahun Tegegn* ("*Gutena*"), (b) Muffed ("*Gugut*"), (c) *Sekel*a ("*Solola*"), (d) *Gelego* ("*Angete melata*"), (e) *Bakelit* (Feathered) and (f) "*Gumaidea*" (Silky feathered)

Study methods

Blood sample collection and DNA extraction: Two hundred and fifty blood samples were collected from different parts of the country (Fig. 2) with a range of 25-30 chicken per sample site. Then, up to 5 mL of the blood sample was drawn in an EDTA coated vacutainer blood collection tube from the wing vein of each chicken. Total genomic DNA was extracted from 10 µL of a blood sample using a Qiagen blood extraction kit as recommended by the manufacturers (www.qiagen.com). The quality and quantity of the extracted DNA were further verified by measuring the absorbance with a spectrophotometer (2000c). The integrity of the DNA was further checked using a gel doc system (GelDoc-It®2 310 imagers) after running the samples on 1% agarose gel electrophoresis.

Polymorphism detection: Initially, the annealing temperature T7 (TAATACGACTCACTATAGGG) and SP6 (ATTTAGGTGACAC TATA) was optimized using a gradient PCR. Then, PCR amplification with 1 µM of 2× bioneer master mix, 0.18 µM of 3 µM forward primer, 0.18 µM of 3 µM reverse primer, 2.5 µM of 25 µM g DNA and the rest of the volume was topped up by ddH_2O to make total reaction volume 50 µL. The PCR conditions setup was 94.0°C for 3:00 min, then 35 cycles of denaturation temperature 94.0°C for 45 sec, annealing temperature 69.0°C for 45 sec, elongation 72.0°C for 1 min and final elongation with 72.0°C for 10 min. The final PCR products were evaluated using 3% of agarose electrophoresis by employing a GelDoc system (GelDoc-It®2 310 imager). Finally, the homozygous samples were directly purified using Qiagen PCR purification kit protocol; heterozygous samples were extracted using the Qiagen gel extraction protocol. All the samples were sequenced using Sanger sequencing.

Genetic diversity analysis: The sequences were trimmed and assembled using CLC main workbench ver7[13]. Multiple sequence alignment was done using the ClustalW algorithm[14,15] and manually edited using MEGA7[16]. The allele size of each haplotype was extracted using sequin application (V 15.5). The genetic distance among groups and the polymorphic information content (PIC) of the marker was analyzed using PowerMarker (V 3.25)[17]. Finally, structure software (V2.3.4)[18] and CLUMPAK[19] were employed to analyze the population structure.

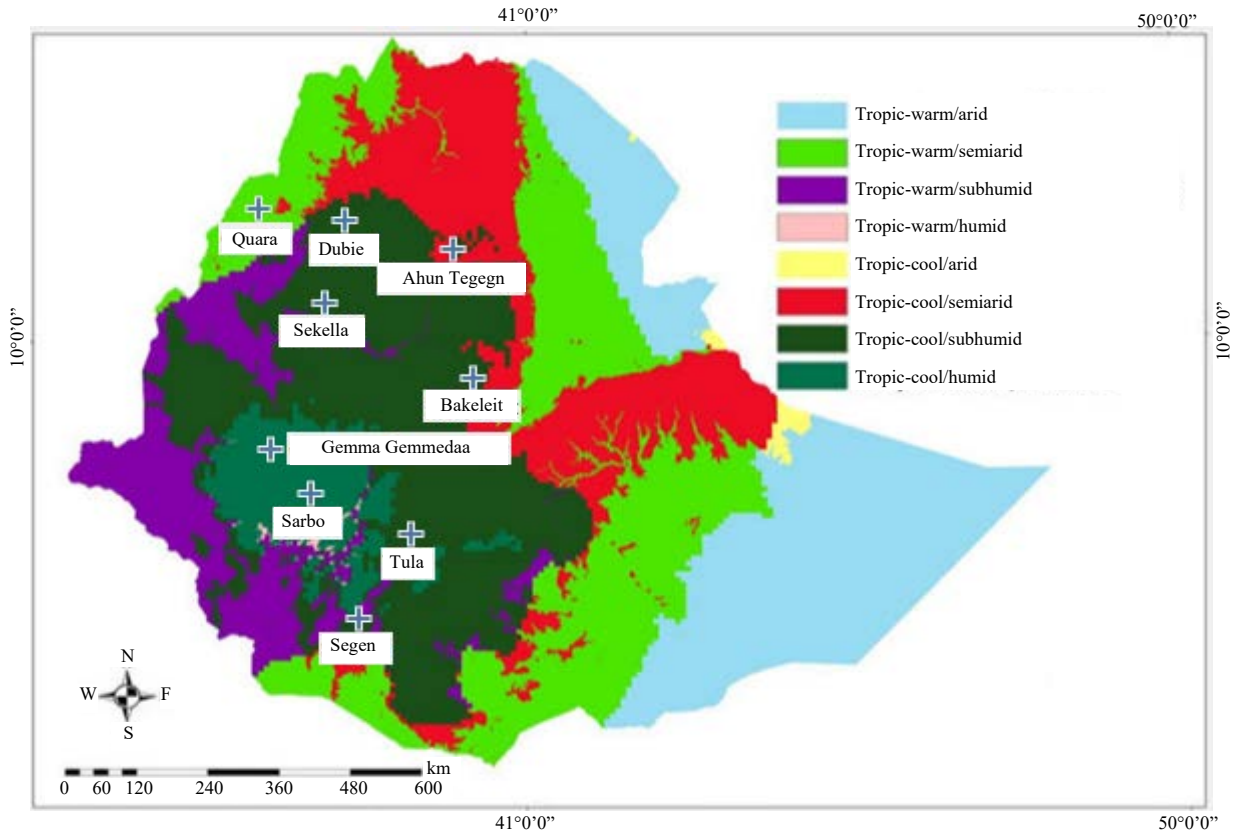

Fig. 2: Map of sample sites, FAO (1996)[11]

RESULTS

Polymorphism in variable number tandem repeats and flanking region of LEI0258: Variable number tandem repeats (VNTR) polymorphism of R12 and R13 in the repetitive region, single nucleotide polymorphism (SNPs) and insertion and/or deletion (indels) in the flanking regions of the microsatellite were considered for the diversity analysis of MHC region[8,9,10,20]. In accordance with this, the VNTRs of R12 and R13 in this study were examined and the result ranged from 3-24 and 1-24, respectively. The repetitive motif of R12 was found more dynamic compared to R13. About 99% of the samples contained only one R13 motif. In regard to the polymorphism in the flanking region, five SNPs sites and 27 indels events were observed both in the upper stream and downstream regions of the marker. The allele size of each haplotype was determined by considering both VNTRs and indels. As a result, 37 kinds of alleles with a range of 193-513 were identified (Table 2) and the PIC of the marker was 0.9288.

Pairwise genetic distance between ecotypes: A total of 112 genotype numbers were identified from 293 sequenced samples. Total genetic diversity within all ecotypes and pairwise genetic distance between groups were calculated from a single locus using Roger's 1972 genetic distance model[21]. As a result, the total genetic distance among the ecotypes was 0.9325. The pairwise genetic distance based on allelic frequency ranged from 0.4950 (between *Gemma Gemmedaa* and *Ahun Tegeng*) to 0.8324 (between *Gumaide* and *Gelego*). Moreover, the genetic distance estimated using the algorithm of the proportion of shared alleles ranged from 0.600 (between *Gumaide* and *Gemma Gemmedaa*) to 0.9545 (between *Sekela* and *Dubie* or *Gelego*) (Table 3). In both approaches, *Gelego* chicken ecotype, which is the naked neck and predominantly found in a dry and warmer part of the country, showed higher genetic distance from others.

Neighbor-joining phylogeny tree: Similarly, the neighbor-joining (NJ) phylogeny tree was constructed based on genetic distance from allelic frequency using Roger's 1972 genetic

Table 2: Variable number tandem repeats in R13 and R12, SNPs and indels polymorphism in the flanking regions of LEI0258 microsatellite

		Upstream	Repetitive		Downstream				
		-30-29/Δ			3	11-18	26	31	Genebank
Haplotype	Size (BP)	TT/Δ	R13	R12	T	ATTTTGAG	A/T	T/A	accession No.
SS16	193	--	1	3		--------			DQ239495
SS19A	195		1	3		--------			MG892209*
TA13	203	--	1	4		--------			MG991120*
GA8B	205		1	4		--------			DQ239505
MG1A	217		1	5		--------			KF534926
GS17	235	--	1	6				A	KF535085
NN4A	237		1	6				A	KF535086
GA8A	249		1	7				A	DQ239513
GB8B	259	--	1	8	C			A	KF535088
DB20B	261		1	8				A	DQ239509
DB18B	271	--	1	9	C			A	KF535089
GA15B	273		1	9				A	KF534932
NN10B	283	--Δ	1	10					KF535090
GB25B	285		1	10				A	KF534934
DB14	295	--Δ	1	11					DQ239496
NN14	297		1	11				A	KF534938
MG23	305	--Δ	1	12			G		KF535093
GB10B	307	--	1	12				A	DQ239550
MG12	308		1	12			T	A	MG892273*
SS22B	309		1	12			T		DQ239494
GA7	319	--	1	13					KF534943
DB15A	321		1	13				A	DQ239552
NN15B	333		1	14				A	DQ239562
GS7	345		1	15				A	DQ239508
DB18A	357		1	16			T		DQ239506
DB20A	369		1	17				A	DQ239530
TA10B	379	--Δ	1	18				A	KF535100
GA12A	381	--Δ	1	18				A	DQ239504
NN19B	393		1	19			T		DQ239493
SJ4	405		1	20			T		DQ239556
MG24	417		1	21			T		MG991170*
GB25A	420		16	5					DQ239548
NN5	427	--	1	22			G	A	MG991145*
NN22	429		1	22			T		MG892263*
SJ17A	453		1	24					MG892343*
MG5B	474		22	3					DQ239499
GA11A	513		25	3					DQ239500

Table 3: Roger 1972 pairwise genetic distances from allelic frequency (lower diagonal) and from the proportion of shared alleles (upper diagonal) between indigenous chicken ecotypes of Ethiopia

ecotypes	*Bakelit*	*Ahun Tegegn*	*Gema Gemmedaa*	*Gumaide*	*Dubie*	*Gelego*	*Sarbo*	*Sekela*	*Tula*
Bakelit	-	0.800	0.7395	0.6695	0.7368	0.7043	0.9231	0.9091	0.7551
Ahun Tegegn	0.5937	-	0.9200	0.8800	0.8674	0.9200	0.8462	0.7945	0.8000
Gema Gemmedaa	0.5382	0.4950	-	0.6000	0.8474	0.9024	0.9500	0.8591	0.9130
Gumaidea	0.6432	0.7400	0.4950	-	0.8947	0.8324	0.9215	0.9091	0.8330
Dubie	0.5526	0.6095	0.6197	0.7621	-	0.9048	0.8704	0.9545	0.8421
Gelego	0.6779	0.7210	0.7560	0.8324	0.7519	-	0.9615	0.9545	0.9524
Sarbo	0.7743	0.5131	0.6442	0.7285	0.7530	0.7793	-	0.9231	0.9231
Sekela	0.6567	0.5745	0.6318	0.7745	0.6603	0.7219	0.5717	-	0.9091
Tula	0.6156	0.4843	0.4989	0.7461	0.6121	0.8178	0.6438	0.5079	-

distance model of power marker software. Various ecotypes sampled in different parts of the country were clustered into three main categories (Fig. 3). Similar to the structure analysis, the *Gelego* ecotype was isolated from the others and formed a separate cluster. However, the rest of the two clusters were of a mixture of ecotypes from different parts of the country. They show a systematic classification fitting neither agro-ecology nor biogeography. The reason might be that ecotypes in high-land and mid-land (also including rift valley) parts of the country have had exposure to similar pathogens or environmental challenges and this has enabled them to share the majority of LEI0258 forms. Moreover, free genetic

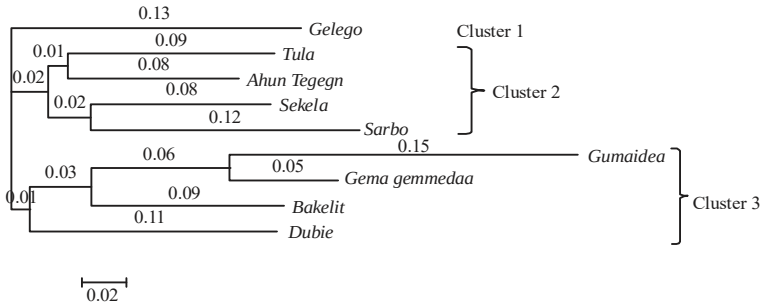

Fig. 3: Unrooted neighbor-joining phylogeny tree based on allelic frequency distance

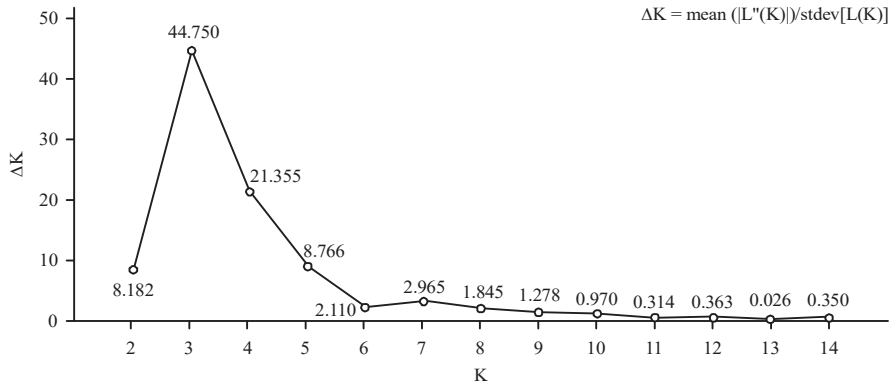

Fig. 4: Delta K value and the probable cluster of Ethiopian indigenous chicken ecotypes distributed across in different agro-ecologies

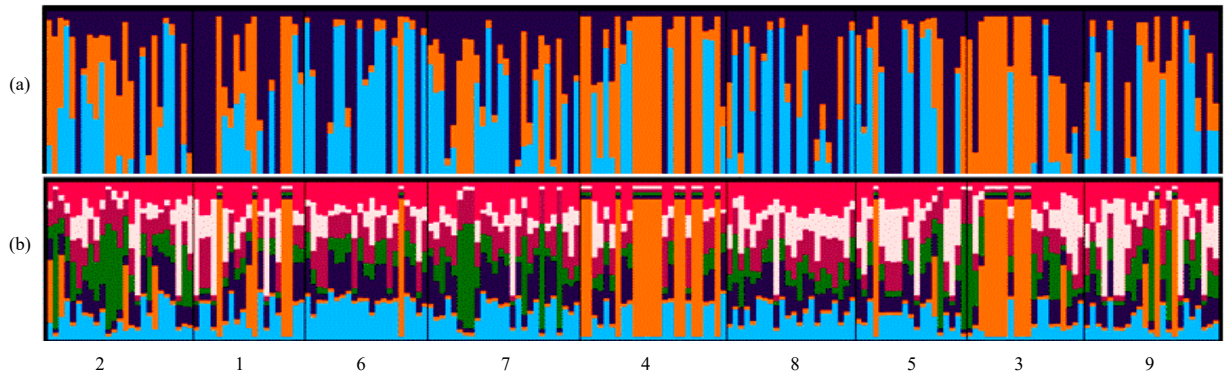

Fig. 5(a-b): The gene pool composition within each ecotypes (a) Δk = 3 and (b) Δk = 7)
1: *Bakelit*, 2: *Ahun Tegegn*, 3: *Gema Gemmedaa*, 4: *Gumaidea*, 5: *Dubie*, 6: *Gelego*, 7: *Sarbo*, 8: *Sekela*, 9: *Tula*

material exchange via trade of chicken between zones or naturally occurring through the free-range scavenging system might play a role in them sharing similar LEI0258 forms.

Population structure analysis: The distribution pattern of indigenous chicken ecotypes was determined based on the percentage of shared alleles across the ecotypes. The possible population group was determined by calculating the optimum evanno K. The best evanno K value was observed at K = 3 (Δk3 = 44.75) as shown in Fig. 4. This indicates that the study populations might have a genetic background to cluster into three different groups just like the neighbor-joining phylogeny analysis, which also produced three clusters as shown in Fig. 3.

The gene pools within each ecotype were observed at Δk = 3 and Δk = 7 (possible grouping) (Fig. 5a-b). As a result,

indigenous chicken found in "*Ahun Tegegn*" and "*Sarbo*" is highly heterogeneous. This indicates that the diversification of MHC regions in these ecotypes are higher than the others. This could be because these groups might have been exposed to a greater variety of disease-causing pathogens than the others. However, the gene pool in "*Segen*" and "*Gema Gemmedaa*" were less diverse. This may be a sampling problem caused by blood sample collection from closely related chickens.

DISCUSSION

Thirty-seven forms of different kinds of alleles ranging from 193-513 were identified from 293 sequenced samples. This result is higher than 26 LEI0258 alleles as identified in North American and European layer-type chicken[8]. The results were also higher compared to 22 and 23 alleles identified in two breeds of Tanzanian chicken[20]. However, this finding is lower than 53 different kinds of alleles discovered from different breeds of Asia, Africa and Europe[9] particularly the 69 alleles found in Chinese indigenous chicken[10].

The polymorphism among haplotypes was evaluated based on VNTR in R12 and R13 motif of repetitive region and SNPs and indels of flanking regions of the marker. Based on VNTR, R12 is more polymorphic than R13. About 99% of the alleles contain only one R13 motif. This result is in line with the findings of Chazara *et al.*[9], in which 23 out of 37 combinations contained only one R13 motif. In addition, five SNPs sites and 27 indels events of the flanking region were observed both in upstream and downstream regions of the marker. With respect to SNPs, this result is aligned with findings in the American and European layer-type chickens, in which two SNPs in upstream and three SNPs in downstream were identified[8]. However, this result is lower than nine and seven SNPs identified in Chinese indigenous chicken by Han *et al.*[10] and seven SNPs identified by Chazara *et al.*[9] in different chicken breeds from Asia, Africa and Europe.

The allelic frequency and shared allele pairwise genetic distance between ecotypes fall within the range of 0.4950-0.8324 and 0.600-0.9545, respectively. This result is lower than the Nei`s 1972 pairwise genetic distance between some ecotypes in the North Western part of Ethiopia as reported by Halima[5]. In contrast, this finding is much higher than the unbiased estimation between chicken ecotypes in Ethiopia using combined microsatellites as identified by Tadelle[6]. Moreover, this finding remains high as compared with the Nei`s pairwise genetic distance between Turkish Denizli chicken sub-populations from 19 microsatellites[22]. A higher value of genetic distance found in the current study might be due to the choice of the genetic distance model and/or the polymorphism nature of the marker that was used.

The neighbor-joining phylogeny tree and structure analysis indicated that the nine ecotypes could be grouped into three main categories. Of that, *Gelego*, the naked neck chicken ecotype from the lowland part of the country forms an isolated cluster. This indicates that this ecotype is genetically far from the feathered chicken. This observation matches the phenotypic information revealed from different parts of Ethiopia by Forssido[23] and Getu *et al.*[12]. In these studies, it was revealed that the naked neck chicken ecotype varied from feathered chicken in terms of body weight and egg production. Similarly, Bodzsar *et al.*[24] found that the naked neck chicken in Hungary formed a distinct cluster from feathered chicken.

CONCLUSION

Since the Ethiopian indigenous chicken is farmed in the harsh, low-input and non-medicated management system, they have been forced to develop various forms of MCH region naturally to survive. As a result, various forms of LEI0258 microsatellite was found in this study, as expected. However, there are some rare alleles which have a restricted distribution. These could be very important alleles and they deserve special consideration for a future breeding program.

SIGNIFICANCE STATEMENT

The study identified a range of forms of the LEI0258 microsatellite marker and found that the marker was highly polymorphic. The majority of the alleles had a wide range of coverage across the country and were found in at least two ecotypes. However, this study also identified some rare alleles, with limited distribution across ecotypes and found with very low frequency. All the major and rare alleles discovered in this study might be important to be considered in a future breeding program. This study will help the researchers to uncover further research on disease tolerance in the indigenous chicken that has not been explored before.

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