PHYLOGENY OF NATIVE CHICKENS IN CENTRAL AND WESTERN MYANMAR*

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Abstract

The present work was conducted to assess the genetic status of some native Myanmar domestic chickens *(Gallus gallus domesticus)* collected from seven different sites in Ayeyarwady, Yangon and Magway Regions from December, 2016 to March, 2017. In this study, the phylogeographic relationships were investigated by analyzing the hyper variable segment (HVS- 1) in mitochondrial DNA of liver tissue sampled from a total of 20 domestic chickens including pygmy chickens. In a 319 bp fragment of HVS-1 DNA among 20 sequences, 33 variable sites that defined 16 haplotypes were identified. Phylogenetic analysis revealed four divergent clades (A, B, C and D) with distinct geographic pattern. The clades A, C, D consist of haplotypes only from Central Myanmar, while the clade B is made up of the haplotypes mainly from the Western Myanmar. Central Myanmar clades (A, C and D) contain the haplotypes mainly related to those of Southwest China and/ or surrounding regions (Japan and Indonesia), whereas the clade B clusters with haplotypes from India, and also from China and Japan. Pygmy chicken haplotype, closely related to one Magway haplotype, together cluster in the clade A. Evolutionary divergence (genetic distance) was lower within each clade of A, B, C and D but higher between the four clades. The distinct geographic pattern in the present phylogenetic tree and network suggests that Myanmar chickens have different geographic origins and have maintained their original geographic diversity in their local domestication history.

Keywords : Native chickens, phylogeny, Myanmar

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Introduction

The domestic chicken (*Gallus gallus domesticus*) provides valuable animal protein for human consumption worldwide (Bhuiyan *et al*., 2013). There are many kinds of native fowls in Asia (Kawabe *et al*., 2014).

Myanmar possesses native chicken populations having well adapted phenotypes to the wet, dry, and hot environments. Farmers in Myanmar, as in many neighboring countries, rear small flocks of native domestic chickens for local consumption.

In contrast to the commercial breeds, the native chicken is mostly maintained in small populations with high genetic diversity and morphological variations (Yap *et al*., 2010).

The modern chicken breeds have lost many of the wild type genes possessed by native chickens. Thus it is very important to retain these wild type genes in the native chicken populations for future utilization in improving genotypes of commercial breeds (Kawabe *et al*., 2014).

Numerous previous works have reported on the phylogeny and origin of the native chickens; some authors postulated single origin for native chickens from the red jungle fowl (*Gallus gallus*), whilst others suggested multiple origins from different species of jungle fowls (Liu *et al*., 2006 in China, Kaginakudru *et al*., 2008 in India, Yap *et al*., 2010 in Malaysia, Sawai *et al*., 2010 in Japan, Ngo Thi Kim Cuc *et al*., 2011 in Vietnam, Bhuiyan *et al*., 2013 in Bangladesh, Miao *et al*., 2013 in China, Hoque *et al*., 2013 in Korea, Kawabe *et al*., 2014 in Laos.

However, phylogenetic works on native chickens have been still rare in Myanmar. In collaborative works, population structure and phylogeny among native chickens were analyzed for populations in Myanmar (Yangon, Pegu, Mandalay) and Indonesia (Aye Aye Maw *et al*., 2012), and for populations in Myanmar, Thailand and Laos (Aye Aye Maw *et al*., 2015).

Mitochondria DNA (mt DNA) has been extensively used as a genetic marker to clarify the phylogeny or maternal ancestral lineage or to compare

populations in native domestic chickens as well as in junglefowls and commercial breeds (Fumihito *et al*., 1996, Kaginakudru *et al*., 2008).

Liu *et al*. (2006), in an extensive work, revealed the existence of nine distinct divergent clades related to the geographic distribution of a wide range of native chickens in Eurasian region.

Hence, the present work was conducted to assess the phylogeography and genetic status of some native chickens in Myanmar with the following objectives: to collect the native chickens from some parts of Western and Central Myanmar and extract genomic DNA, to find out genetic variation among the collected specimens based on HVS-1 mtDNA control region marker sequence, and to relate genetic variation to sample collection sites via molecular phylogenetic analysis.

Materials and Methods

Chicken sample collection and study period

A total of 20 native chickens *Gallus gallus domesticus* (Linnaeus, 1758), were collected live from seven different sites: Pathein, Ngwesaung, Tagongyi, Myaungmya in Ayeyarwady Region; Twantay and Hmawbi in Yangon Region ; and Magway in Magway Region of Myanmar. This study was conducted from December, 2016 to March, 2017. The laboratory work was conducted at the Molecular biology Laboratory at the Department of Zoology, University of Yangon.

Liver tissue sampling

Small pieces of liver tissue (1 gm each) were collected from each chicken (n = 20). The liver tissue samples were preserved in 70% ethanol in tissue sample tubes with caps.

Genomic DNA extraction

Total genomic DNA was extracted from 25 mg liver tissue (n =20) by Invitrogen Purelink Genomic DNA extraction Minikit (10), USA. The protocol followed was as directed by the kit manufacturer. Amount and purity of the extracted DNA were measured for each sample (n = 20) by NanoDrop One spectrometer

PCR amplification of HVS – 1 DNA

HVS – 1 DNA fragment (550 bp) was amplified by the set of primers L16750 (F) and H522 (R). (Fumihito *et al*., 1994; Fu *et al.*, 2001). AmpliTag Gold 360 Master Mix was utilized.

The PCR running conditions were as follows :

Hold	95^{o}C	5 min
Denature	94^{o}C	1 min
Annealing	60^{o}C	1 min
Extension	72^{o}C	1 min
Final extension	72^{o}C	7 min

The PCR was run for 30 cycles in 20μL reaction

Sequencing PCR reaction

The amplified HVS-1 DNA fragment (550 bp) was utilized for sequencing reactions. Using the same primers: L16750 (F) and H522 (R) for sample template. The control primers – 21 M13 were used for pGEM-3zf(+) Control template. Big Dye Mix was utilized.

The running conditions for the sequencing PCR were as follows:

Hold	96^{o}C	1 min
Denature	96^{o}C	10 sec
Annealing	50^{o}C	5 sec
Extension	60^{o}C	4 min
Holding	4^{o}C	finite

The PCR was run for 25 cycles in 10.5 μL reaction

Sequencing in ABI 3500 Genetic Analyzer

A total of 20 HVS- 1 amplified DNA marker were sequenced and read for both forward and reverse primer extended template strands. The sequenced data were downloaded from ABI3500 Computer onto CD discs and Laptop computer using MEGA 7 software and the sequences were aligned by Proseq for 319 bp out of 550 bp amplified.

Agarose gel electrophoresis

Agarose gel electrophoresis were ran to check extracted genomic DNA and PCR amplified HVS-1 DNA of mitochondria. Agarose gel (1%) in TAE 1 x buffer (pH 8.0). Gel was run in TAE 1 x buffer at 135 VDC for 10 min – 15 min. The gel was stained with ethidium bromide (10 mg/L) for 30 min in distilled water, washed in tap water, and DNA bands were visualized and photographed under long wave UV light on a transilluminator.

Data analysis

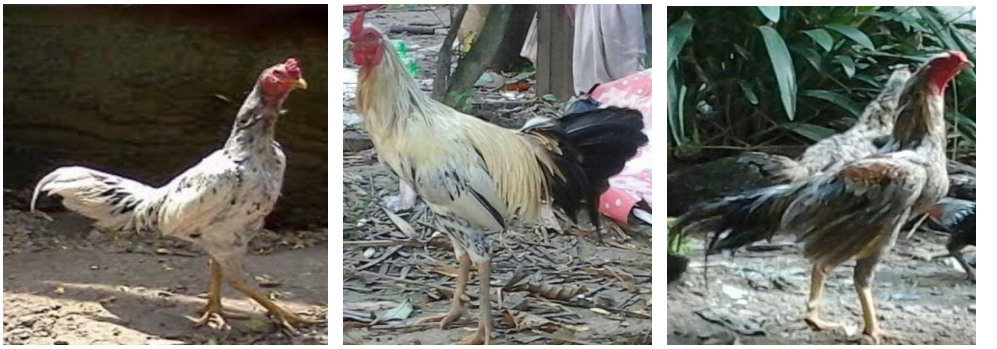
A Maximum Likelihood (ML) phylogenetic tree and a Median-Joining network were constructed for 20 aligned haplotype sequences (319 bp) of HVS – 1 DNA marker from 20 collected chickens of different sites. Reference sequences for domestic chicken, (*Gallus gallus domesticus*) deposited in GenBank Database, were downloaded and compared to the sequences of the present study. Haplotype diversity, nucleotide diversity, variable sites and genetic distances were also calculated. MEGA 7 software, Proseq software, Arlequin ver 3.5 were utilized.

Results and Discussion

Morphological diversity such as variations in plumage colour and body size (e.g dwarfs) occur among Myanmar native domestic chickens. However, little is known about the origins and phylogeography of native chickens in different parts of Myanmar. Hence, the present study was conducted to assess the genetic status of some Myanmar native chickens.

In the present study, a mitochondrial DNA (mt DNA) D-loop Hyper Variable Segment (HVS-1) sequences (319 bp) were analyzed and compared among 20 sequences of Myanmar native chickens collected from seven different sites (populations) in Central and Western Myanmar (Figure.1, Table 1).

A. TS7, Myaungmya B. TS11, Pathein C. TS14, Tagongyi



D. TS16, Ngwesaung E. TS17, Magway F. TS21, Hmawbi

G. TS25, Twantay

Figure1. Some studied native chickens from different collection sites

Table 1 . Haplotypes and accession numbers of chicken mtDNA sequences used in this study

Haplotype		GenBank accession No.	Reference
A- TS15,TS 19, TS 21,TS24, TS 25, TS 26			This study
B- TS11,TS 12, TS 13, TS14, TS 16, TS 17 TS 22, TS 23			This study
Haplotype		GenBank accession No.	Reference
C- TS 7,TS 8, TS 9, TS18			This study
D- TS 10, TS 20			This study
G. gallus domesticus	Yunan, China	AF512057	GenBank
G. gallus domesticus	Sichuan, China	AF51206	
G. gallus domesticus	Yunan, China	AY392172	
G. gallus domesticus	India	AY644966	
G. gallus domesticus	Ryuku, Japan	AB007744	
G. gallus domesticus	Japan	AB268535	
G. gallus domesticus	Japan	AB268543	
G. gallus domesticus	H 10, China	AY588636	
G. gallus domesticus	Shizuoka, Japan	AB114076	
G. gallus domesticus	Shizuoka, Japan	AB114070	
G. gallus domesticus	Ibaraki, Japan	AB114069	
G. gallus domesticus	Cuanzi, China	AF512285	
G. gallus domesticus	Cuanzi, China	AF512288	
G. gallus	Vietnam	AB009434	
G. gallus	Japan	D82904	Out group
G. gallus	Indonesia	AB268545	Out group

Among studied chickens, a total of 33 variable sites (i.e nucleotide substitutions) that defined 16 mthaplotypes were found among the 20 sequences from seven populations of Myanmar. Overall haplotype and nucleotide diversity were 0.9737 ± 00250 and 0.027207 ± 0.016667 respectively for the total 20 native chickens studied (Tables 2 and 3).

These results indicated that studied Myanmar native chickens have higher haplotype and lower nucleotide diversities although they have moderate nucleotide polymorphism (variable sites) compared to Laotian native chickens which have 37 variable sites, 29 haplotypes, and haplotype diversity of 0.8798 and nucleotide diversity of 0.10158 among three populations (Kawabe *et al.,* 2014). In another work on four populations among Bangladeshi native chickens, 39 variable sites. 29 haplotypes, 0.901

haplotype and 0.016 nucleotide diversity respectively were reported by Bhuiyan *et al.* (2013).

Table 2. Variable sites between studied sequences

	Variable Sites																														
	1	3	3	4	6	8	9	1 2	1 3	1 3	1 4	1 4	1 5	1 5	1 6	1 6	1 7	1 7	1 7	1 8	1 8	2 0	2 1	2 2	2 3	2 3	2 3	2 5	2 6	2 7	3 1
	6	2	8	1	0	8	8	0	3	8	3	6	5	7	4	7	0	5	7	2	6	2	7	7	1	4	6	8	1	3	6	4	7
TS7Myaungmya	A	G	T	T	A	T	A	T	G	T	A	C	T	C	C	C	A	C	C	T	C	A	C	T	T	C	T	C	C	G	T	C	T
TS8Myaungmya	A	T	T	T	A	T	A	T	G	T	A	C	T	C	C	C	A	C	C	T	C	A	C	T	T	C	T	C	C	A	T	C	C
TS9Myaungmya	A	T	T	T	A	T	A	T	G	T	A	C	T	C	C	C	A	C	C	T	T	A	C	T	T	C	T	C	C	G	T	C	T
TS10Myaungmya	A	T	T	T	A	T	A	T	A	T	A	C	C	T	C	C	A	T	C	T	C	G	C	C	T	C	C	A	C	G	T	C	T
TS11Pathein	A	T	T	T	A	T	A	C	G	C	A	C	C	T	C	C	A	T	C	T	C	A	C	T	T	C	C	A	C	A	T	C	T
TS12Pathein	T	T	T	T	T	T	A	T	G	C	G	C	C	T	C	C	A	T	C	T	C	A	C	T	T	C	C	A	T	A	T	C	T
TS13Pathein	A	T	T	T	A	T	A	T	G	C	A	C	C	T	C	C	A	T	C	T	C	A	C	T	T	C	C	A	C	G	T	C	T
TS14Tagonegyi	A	T	T	T	A	T	A	C	G	C	A	C	C	T	C	C	A	T	C	T	C	A	C	T	T	C	C	A	C	A	T	C	T
TS15Tagonegyi	A	T	T	C	A	C	A	T	G	T	A	T	C	T	T	C	A	T	T	C	C	A	C	T	C	C	C	A	C	A	T	C	T
TS16Ngwesaung	A	T	T	T	A	T	A	T	G	C	G	C	C	T	C	C	A	T	C	T	C	A	C	T	T	C	C	A	T	A	T	C	T
TS17Magwae	A	T	T	T	A	T	A	C	G	T	A	C	C	T	C	C	A	T	C	T	C	A	C	T	T	C	C	A	C	A	T	C	T
TS18Magwae	A	T	G	T	A	T	A	T	G	T	A	C	T	C	C	C	A	C	C	T	C	G	C	T	T	T	T	C	C	A	T	C	T
TS19Magwae	A	T	T	T	A	T	A	T	A	T	A	C	C	T	T	T	A	T	T	C	C	A	T	T	C	C	T	A	C	A	T	C	T
TS20Magwae	A	T	T	T	A	T	T	T	G	T	A	C	C	T	C	C	A	T	C	T	T	G	C	C	T	C	C	A	C	G	T	T	T
TS21Hmawbi	A	T	T	T	A	C	A	C	G	T	A	T	C	T	T	C	A	T	T	C	C	A	C	T	T	C	C	A	C	A	T	C	T
TS22Hmawbi	A	T	T	T	A	T	A	T	G	C	G	C	C	T	C	C	G	T	C	T	C	A	C	T	T	C	C	A	C	A	C	C	T
TS23Hmawbi	A	T	T	T	A	T	A	T	G	C	G	C	C	T	C	C	G	T	C	T	C	A	C	T	T	C	C	A	C	A	C	C	T
TS24Hmawbi	A	T	T	T	A	C	A	C	G	T	A	T	C	T	T	C	A	T	T	C	C	A	C	T	C	C	C	A	C	A	T	C	T
TS25Twantay	A	T	T	T	A	T	A	T	A	T	A	C	C	T	T	T	A	T	T	C	C	A	T	T	C	C	T	A	C	A	T	C	T
TS26Twantay	A	T	T	T	A	T	A	T	A	T	A	C	C	T	T	T	A	T	T	C	C	A	T	T	C	C	T	A	C	A	T	C	T

Table 3. Polymorphic sites, haplotype and nucleotide diversity of four clades

Clade	n	No. of haplotypes	No. of polymorphic sites	Haplotype diversity (SD)	Nucleotide diversity (SD)
A	6	4	9	1.0000 (0.0962)	0.015047 (0.009873)
B	8	6	9	0.9286 (0.0844)	0.010636 (0.006925)
C	4	4	7	1.0000 (0.1768)	0.011494 (0.008734)
D	2	2	4	1.0000 (0.5000)	0.012539 (0.014019)
Total	20	16	33	0.9737 (0.0250)	0.027207 (0.014647)

Clade A: TS15, TS19, TS21, TS24, TS25, TS26
Clade B: TS11, TS12, TS13, TS14, TS16, TS17, TS22, TS23
Clade C: TS7, TS8, TS9, TS18
Clade D: TS10,TS20

Phylogenetic analysis, based on ML tree and Median- Joining network, (Figs. 2 and 3), revealed that four distinctly divergent clades (A, B, C and D) with distinct phylogeographic pattern, occurred among the seven different native chicken populations of Myanmar studied in the present work. Clades A, C and D consist of haplotypes from Central Myanmar related to mainly Chinese haplotypes. Clade B is composed of haplotypes mainly from Western Myanmar related to Indian haplotype. Interestingly, the Magway haplotypes are distributed in all four clades of the studied Myanmar native chickens, indicating gene flow or genetic admixture between the Magway population and the rest of the studied native chickens in Central and Western Myanmar (Fig. 3).

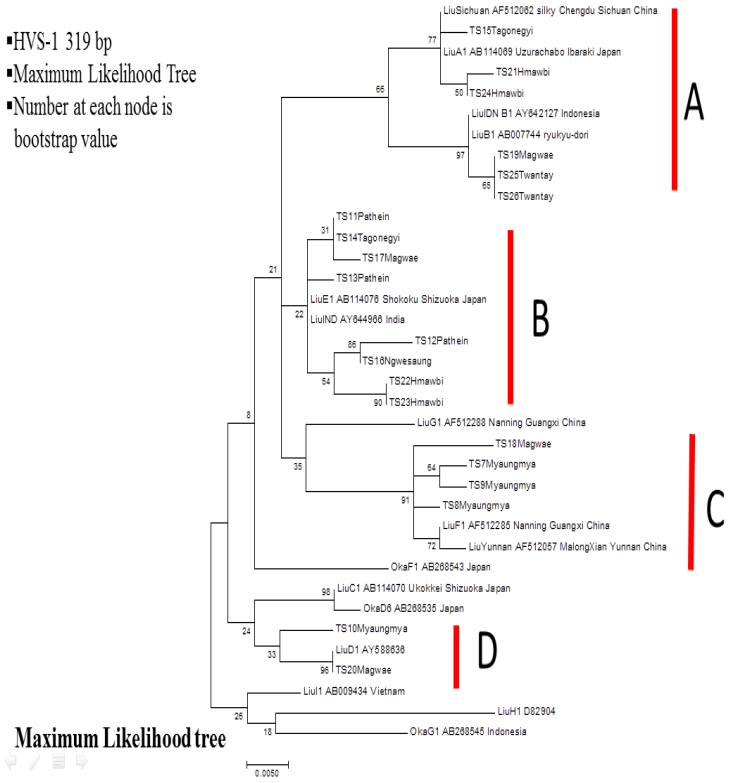


Figure 2. Maximum Likelyhood Phylogenetic tree constructed using HVS-1 data of the studied native chicken (n = 20) of Myanmar. Scale indicates number of nucleotide substitutions per site. Reference sequences are from GenBank.

Median-joining network analysis also showed that the native chicken haplotypes of Myanmar in clade B were also related to a Japanese haplotype indicating some genetic admixture from the northern areas of Myanmar (Fig. 3).

The pygmy or dwarf native chickens from Twantay are found to be closely related to one Magway haplotype, clustering together in clade A (Figs. 1, 2 and 3).

Laotian native fowls were distributed across five clades with mostly in two clades originated in China; the other three clades were reported to be probably originated in Southeast Asia (Kawabe *et al.*, 2014).

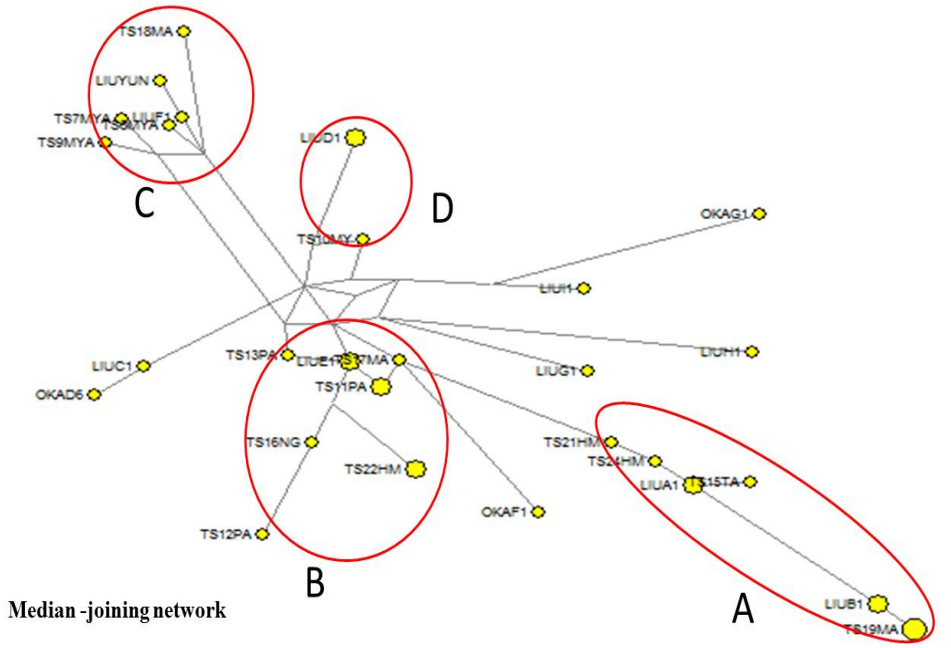


Figure 3. Median – joining network of HVS-1 sequencaes (n = 20) among the studied native chickens. Circle (yellow) size indicates haplotype frequency. Reference sequences are from GenBank.

Median – joining network of mtD-loop (648 bp fragment) revealed five clades with mostly in two clades among Bangladeshi native chickens;

they were thought to originate particularly from Myanmar and China (Bhuiyan *et al.*, 2013).

Regarding genetic distance (evolutionary divergence, expressed as number of substitutions of nucleotides per sequence site), between native chickens (Pathein, Hmawbi and Magway) and an Indian native chicken ranged 0.013 to 0.036, whereas, between Myanmar native chickens and a Chinese native chicken ranged 0.013 to 0.046. This indicated that native Western Myanmar chickens (clade B) are genetically closer to Indian native chicken than to Chinese native chickens (Table 4). But the gene flow seemed to be from Myanmar to neighbouring countries. This assumption is based on the results of work by Aye Aye Maw *et al.* (2015) who stated that genetic admixture (gene flow) was observed between Thai and Laotian native chickens, while foreign gene admixture was not found among Myanmar native chickens in Yangon, Pegu and Mandalay. The above researcher group also reported that the Neighbour-joining tree analysis showed Thai and Lao native chickens closely clustered in a single clade whereas Myanmar native chickens diverged into a different clade.

Table 4. Genetic distances between foreign and local native chickens

TS 13 Pathein	vs	India	0.013
TS 13 Pathein	vs	China	0.040
TS 24 Hmawbi	vs	India	0.036
TS 24 Hmawbi	vs	China	0.046
TS 18 Magway	vs	India	0.029
TS 18 Magway	vs	China	0.013

Aye Aye Maw *et al.* (2012) found that the genetic distance was 0.088 among native chicken populations of Myanmar and Indonesia.

Results of the present study show that genetic distance among studied Myanmar native chickens ranged 0.036 to 0.050 indicating moderate genetic diversity with less gene flow among the seven native Myanmar chicken populations studied which were diverged into four different clades with distinct geographic patterns (Figs. 2 and 3, Table 5).

Table 5. Genetics distances between local native chickens

TS 24 (Hmawbi)	vs	Indonesia	0.050
TS 24 (Hmawbi)	vs	TS 18 (Magway)	0.050
TS 15 (Tagongyi)	vs	TS 18 (Magway)	0.050
TS 9 (Myaungmya)	vs	TS 15 (Tagongyi)	0.046
TS 7 (Myaungmya)	vs	TS 24 (Hmawbi)	0.046
TS 18 (Magway)	vs	TS 25 (Twantay)	0.046
TS 18 (Magway)	vs	TS 12 (Pathein)	0.043
TS 26 (Twantay)	vs	TS 7 (Myaungmya)	0.043
TS 12 (Pathein)	vs	TS 9 (Myaungmya)	0.040
TS 26 (Twantay)	vs	TS 16 (Ngwesaung)	0.036

Evolutionary divergence estimates among the four clades of the studied Myanmar native chickens revealed that the divergence was low and similar, at 0.01 substitution of nucleotide per site of HVS-1 sequence (319 bp), within each clade of A, B, C and D. On the other hand, the divergence between the four clades ranged. 0.025 to 0.044, suggesting higher genetic diversity between the four clades compared to low diversity within each clade (Tables 6 and 7).

Table 6. Estimates of average evolutionary divergence over sequence pairs within clades

Clade	Average Evolutionary Divergence
Clade A	0.0154
Clade B	0.0108
Clade C	0.0117
Clade D	0.0128

Table 7. Estimates of evolutionary divergence over sequence pairs between clades

	A	B	C
Clade A			
Clade B	0.032		
Clade C	0.044	0.032	
Clade D	0.039	0.025	0.034

It is hoped that the data generated and the conclusion reached in the present work would contribute to future research on the phylogenetics of the native chickens of Myanmar.

Conclusion

Overall data of the present phylogenetic analysis revealed four divergent clades with distinct geographic patterns occurred among the studied native Myanmar chickens collected from seven different collecting sites or seven populations. Myanmar native chickens have different geographic origins within the country. Myanmar native chickens have thus maintained their original geographic genetic diversity in their local domestication history.

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