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Molecular Characterization of Nepalese Indigenous Chicken, Sakini, Based on Mitochondrial DNA Displacement (D)-loop Sequences

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Abstract

This study is the first time to perform molecular characterization in indigenous chickens, Sakini, of Nepal for studying genetic diversity and its relationship with its assumed progenitors. The first 522 nucleotides of hypervariable I (HVI) segment of the D-loop from 33 individuals were PCR amplified and subsequently sequenced. Fourteen haplotypes out of 33 sequences were identified from 20 polymorphic sites. Haplotype (gene) diversity (Hd) is 0.813 with SD 0.065 and nucleotide diversity (Pi) is 0.00525 with SD 0.00091. The neighbour joining tree indicated that Red Jungle Fowl from India is the progenitor of the Nepalese Sakini chicken. NETWORK analysis revealed that it can be grouped into four distinct Haplogroups (A1, E1, E2, and E3) respectively. Seventeen individuals belonged to E1, eight to E3, seven to E2, and one to A1. The high mitochondrial D-loop diversity in Nepalese Sakini chicken with multiple maternal origins serves the scientific basis for the development of rational policies supporting conservation efforts and provides directions for future research for developing sustainable genetic improvement approaches.

Keywords: Nepalese indigenous chicken, mitochondrial DNA, D-loop, haplotype, phylogenetic analysis, haplogroup

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Introduction

Poultry farming has become one of the important businesses in both rural and urban areas of Nepal. The chickens of indigenous breeds have both social and cultural values. Backyard poultry farming also aids in reducing poverty in rural areas of the country. As a result, the percentage of indigenous poultry exceeds that of commercial chickens from exotic breeds in such areas. Some of the documented indigenous chicken breeds of Nepal are Sakini, Ghati Khulie and Pawkh Ulte. These native breeds share diverse phenotypic and genotypic variations [1]. Indigenous breeds are valuable assets as they serve as a major pool for genetic diversity [2].

As the utilization of commercial chickens has been increased since a few decades due to their faster growth and higher production rates, the population of indigenous chickens has sharply decreased [3]. Furthermore, the consumption of native chickens is neglected by the new generation of people nowadays as these chickens are thought to have a slower growth rate and lower production performance [4], and, moreover, their meats are assumed to be tougher. These indigenous chickens in the true state therefore are difficult to find due to indiscriminate breeding and intermixing with the commercial breeds, which can reduce the potential for unique adaptation of the local chickens to existing harsh conditions [5]. This scenario thus has led in the loss of beneficial alleles from the gene pool and brings about the genetic erosion of village chickens, which subsequently causes decreased genetic variability and potential in indigenous breeds. It is therefore important to identify unique indigenous chicken genetic resources in order to sustainably utilize and improve them towards disease resistance to localized pathogens and adaption to ongoing climate change driven environmental conditions.

There are two major traditional approaches for assessing genotypic diversity: Cytogenetics and morphological studies [4,6]. However, molecular techniques such as mitochondrial DNA (mtDNA) and microsatellite loci markers as well as single nucleotide polymorphisms (SNPs) are widely used nowadays for investigating genetic diversity [7]. In order to investigate the genetic relationship between the closely related species, mtDNA has been widely used [8]. Moore (1995) suggested that mtDNA has a faster evolution rate than nuclear DNA [10]. The evolution rate of mtDNA is 5-10 times that of nuclear DNA fragments. More importantly, the non-

coding control region of mtDNA which comprises the most variable sites in displacement loop (D-loop) is primarily used to analyse the genetic origin of animals and also to investigate the domestication process for livestock species [11,12].

Even though indigenous chickens have significant contribution to country's economy, the Nepalese indigenous chickens are not adequately explored and genetically characterized [1]. Therefore, it is utmost important to characterize the Nepalese indigenous chickens at molecular level. The present study was designed to investigate the origin and genetic diversity within the principal indigenous chicken breed, Sakini chicken, using mtDNA control region sequence variation. In addition, many Nepalese scientists claim these Sakini chickens to be related to Red Jungle Fowls called *Luinche*, however, the scientific evidence is lacking. This study also tried to determine the relatedness of the Red Jungle Fowls and the Nepalese Sakini chickens.

Materials and methods

Sampling

Thirty-three blood samples from Sakini chickens of base population were collected from three different agro-ecological zones viz. Terai, mid-hill and high-hill. Since Nepal has three agro-ecological zones, the chosen samples from these three agro-ecological zones will give overview of the country. Then, the genomic DNA was extracted from each sample following manufacture protocol (Promega DNA extraction kit, Catalogue number: A1120). The quality of each DNA sample was checked by using a Nanodrop spectrophotometer (Thermo Scientific™ NanoDrop 2000). Then, the DNA samples were kept at -20 degree Celsius for future use. Forty [40] reference sequences belonging to 19 haplogroups and 18 jungle fowl sequences were retrieved from GenBank, NCBI. These references cover most of the haplotypes and jungle fowls that are found neighboring countries such as India, China, Myanmar, and Indonesia.

Table 1. Accession number of Red Jungle Fowls and domestic chickens representing different haplogroups retrieved from GenBank, NCBI

S.N.	Accession number	Haplogroups	References
1	GU261684.1, GU261695.1	A	[13]
2	GU261704.1 (*G. g. spadiceus*, Yunnan, China], AP003321.1 (Red Jungle Fowl *G. g. spadiceus*, Laos)	B	[13]
3	KX987152.1	B3	[14]
4	AF128344.1, GU261679.1, GU261718	C1	[13]
5	GU261680.1	C2	[13]
6	GU261707.1, GU261716.1	C3	[13]
7	AB009436.1 Red Jungle Fowl *G. g. gallus*, Indonesia)	D	[13]
8	GU261683.1	D2	[13]
9	GU261677.1, GU261697.1	D3	[13]
10	GU261694.1, GU261713.1, AY235570.1, GU261686.1, HQ857210.1, GU261712.1, AP003580.1	E1	[13]
11	HQ857209.1	E2	[13]
12	GU261708.1	E3	[13]
13	GU261711.1, GU261702.1, GU261703.1, GU261688.1, GU261717.1, DQ648776.1	F	[13]
14	GU261690.1, GU261678.1, GU261676.1	G	[13]
15	GU261715.1	H	[13]
16	GU261698.1	I	[13]
17	GU261692.1 (*G. g. spadiceus*, Yunnan, China)	X	[13]
18	GU261693.1 (*G. g. spadiceus*, Yunnan, China)	Y	[13]
19	GU261674.1 (*G. g. jabouillei*, Hainan, China)	Z	[13]

Table 2. Accession number and their assigned haplotypes of Red Jungle Fowls from different countries retrieved from GenBank, NCBI

S.N.	Accession no.	Countries	References
1	GU261707.1(C3), GU261708.1(E3), GU261709.1(E1) ; KP211423.1	India	[13]
2	GU261706.1(W), GU261692.1(X), GU261693.1(Y), GU261704.1(B), GU261695.1(A), GU261696.1(Z), GU261674.1(Z), GU261702.1(F),	China	[13]
3	NC_040902.1, GU261716.1(C3), GU261703.1(F)	Myanmar	[13]
4	Red Jungle Fowl (*G. g. gallus*; AB007720.1(K02))	Palembang, Indonesia	[13]
5	Red Jungle Fowl (*G. g. bankiva* ; AB007718.1)	Bali, Indonesia	[13]

PCR amplification and sequencing

Two conserved primers, L16750 and H547, were used for amplifying 522 base-pair (bp) long hyper-variable I (HVI) segment of the D-loop of chicken mtDNA. The oligonucleotide sequences of L16750 is 5′-AGG ACT ACG GCT TGA AAA GC-3' and H547 5′-ATG TGC CTG ACC GAG GAA CCA G-3' (Niu et al., 2002). The component of 2X master mix includes bacterially derived *Taq* DNA polymerase, dNTPs, $MgCl_2$, and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. A 25 µl of PCR amplification reaction mixture was prepared by keeping 13 µl of PCR 2X Master Mix, 5 µl of template DNA, 5 µl of molecular grade nuclease-free water, and 1.5 µl of forward and 1.5 µl of reverse primers. Subsequently, PCR tubes containing the mixture were kept in a PCR thermocycler. The PCR condition was as follows: denaturation at 95°C for 1 minute, annealing at 63°C for 1 minute and extension at 72°C for 2 minutes. The PCR products were run on 1.5% agarose gel. The visualization of DNA bands of agarose gel were done under UV gel documentation by comparing with a DNA molecular weight marker (100 bp ladder). Then 10 µl of the PCR product of each sample was sent to the Department of Animal Genetic Resources, National Livestock Research Institute, Rural Development Administration, Korea for sequencing. The obtained D-loop sequences were used for investigating genetic diversity.

Data analysis

The raw chromatogram files of D-loop sequences of the 33 samples were edited using BioEdit software and aligned using ClustalW program [15] built in MEGA 5 software [16]. At first, we constructed an unrooted neighbour-joining (NJ) phylogenetic tree following Kimura-2-parameter model using the MEGA and then DnaSP V.5.10 program was used to calculate variable sites, haplotype diversity [Hd) and nucleotide diversity (Pi) [17]. In order to investigate the possible relationships among the sequences of each major clade in the NJ tree, median-joining networks were constructed using the program Network 3.1 [18].

Results and Discussion

Molecular technology development has increased the efficiency and accuracy in the genetic characterization of breeds of domestic animals. Only a few studies on molecular characterization were performed in different livestock breeds of Nepal, to evaluate their genetic diversity and functional genomics [12–14]. The present study aimed to carry out molecular characterization in Nepalese Sakini chickens using the mtDNA displacement (D)-loop sequences to examine their origin and genetic diversity.

Mitochondrial DNA D-loop variability in Sakini chickens

The sequences of the first 522 nucleotides were used for analysis. The Nepalese Sakini chicken sequences have shown a high variability at haplotype level. Fourteen haplotypes out of 33 sequences were identified from 20 polymorphic sites with polymorphisms between 167 and 447 nucleotide positions. Out of the 20 polymorphic sites, 13 are singleton variable sites and seven are parsimony informative sites with two variants. While comparing with the GenBank references *Gallus gallus* mtDNA sequences of a Tibetan chicken (NC_040970.1; [19] and a White Leghorn chicken (NC_001323, Valverde et al., 1994), there is no indel between Tibetan and Nepalese Sakini chickens but there is a triple-nucleotide (CCC) insertion starting at the 50th and ending at 52th position of the mtDNA genome (16775 bp) and this triple-nucleotide segment is also present in the Red Jungle Fowl (AB007720.1: *G. g. gallus*) of Southeast Asia, that is claimed to be the ancestor of the Asian domesticated chickens. The functional importance of this indel has not been investigated. Furthermore, a very unique transitional mutation (G/A) at the 249th position was observed among Nepalese Sakini chicken sequences which might be related to some functional phenotypes such as strong adaptation to adverse environments or disease resistances [19]. This mutation (G/A) is also found in Javanese Red Jungle Fowl (*G. g. bankiva*, AB007718.1) of Southeast Asia.

Phylogenetic analysis of Nepalese Sakini chickens

MtDNA study provides valuable information on the origin and diversity of domestic chicken populations [20]. Miao et al. (2013) re-evaluate global mtDNA profiles of Red Jungle Fowls and domestic chickens and describe the distribution of the haplogroups (HGs) of both wild Red Jungle Fowls and domestic chickens. Among the nine global HGs (A-I), seven are shared by Red Jungle Fowls and domestic chickens (A, B, C, D, E, F, and G) while six of them (except D) are dominant HGs with relatively wider geographic distributions[13]. The mtDNA phylogeny of Nepalese Sakini chickens is divided into four distinct sub-HGs (A1, E1, E2, and E3) when the 14 Sakini chicken haplotypes were alignedagainst these previously defined HGs (**Figure 1a**). These results demonstrate a high mtDNA D-loop diversity with at least two major maternal origins for Nepalese Sakini chickens.

1a

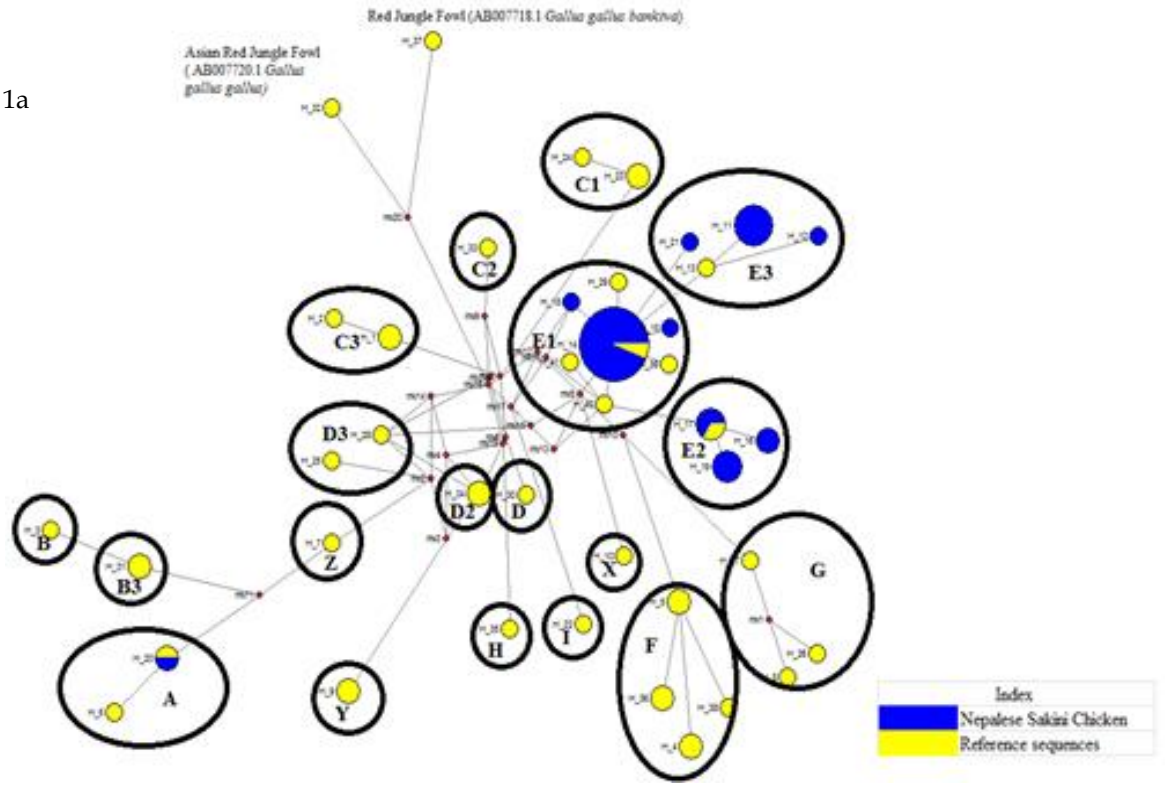

Figure 1. 1a: Network profile of major haplogroups present in Nepalese Sakini chickens against 40 reference sequences from the GenBank representing the 13 haplogroups (e.g., HG A1, E1, E2, and E3); 1b: Unrooted neighbour joining tree from mtDNA D-loop sequences of Sakini chickens against Red Jungle Fowls in the region

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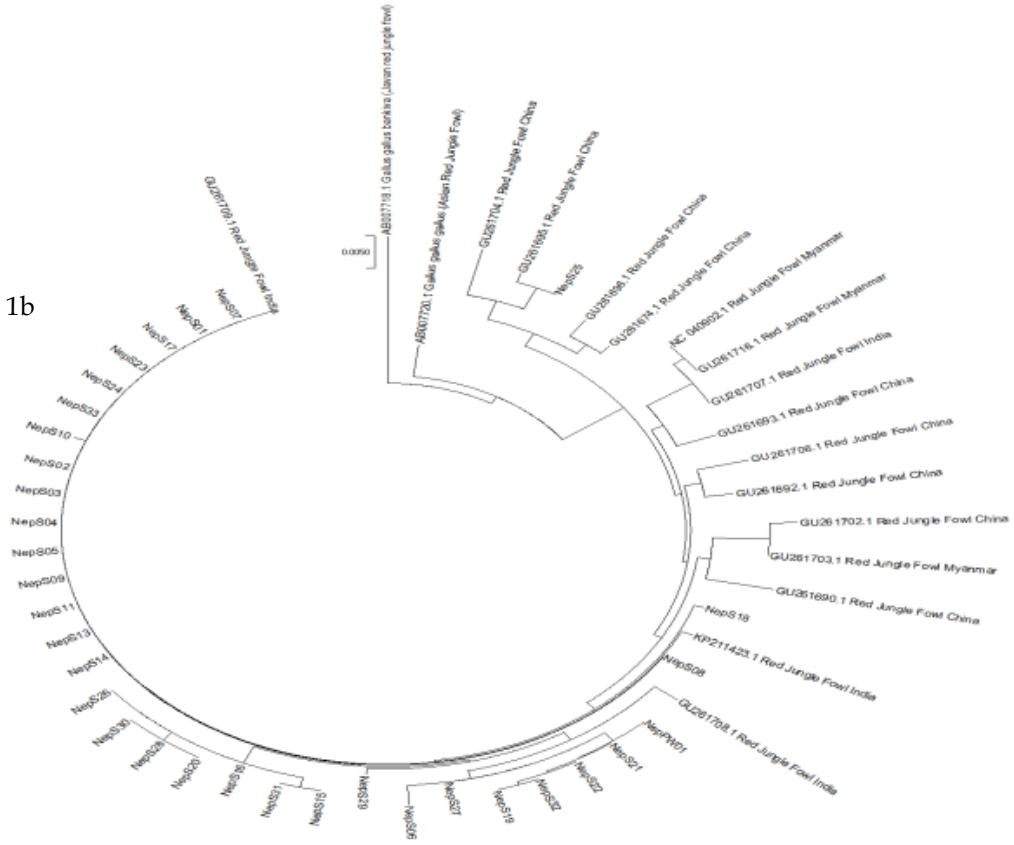
1b



Seventeen individuals belong to sub-HG E1, eight to E3, seven to E2, and one to A1. It is not surprising to have 96% of the Nepalese Sakini chickens to be classified into HG E as the HG E dominates in Europe, Middle East, and South Asia, and more specifically, around 56% of indigenous chickens of India, with whom Nepal has free border, fall into in the HG E [13]. Furthermore, sub-HG E1 is widely distributed in all geographically defined populations whereas its sister sub-HGs E2 and E3 are mostly found in South Asia [21]. The findings of our study also support the claim that the root of the HG E is in the Indian sub-continent.

Presence of HG A in Nepalese Sakini chickens might be attributed to the possible gene flow from indigenous chickens of Yunnan province, China as the high proportion of HG A is found there, claiming the origin of the HG A to be in Yunnan and/or surrounding areas. The pattern or gene flow specifically for the HG A can be explained by the easy transportability of chickens that were carried by humans across the world during migrations or across the trade routes (e.g., from Tibet, China to Calcutta, India) throughout the history. The samples, however, were collected from base population of Sakini chickens only from central Nepal covering the narrow area of the country which does not give any clear picture of the contribution of Nepalese chicken genetic resources to the global scenario, thus further investigation is necessary to involve chicken samples from a wider geographical distribution.

Relatedness of Nepalese sakini chickens with their wild progenitors

Nepalese scientists claim the indigenous chickens in the country to be related to Red Jungle Fowl. The current comparison of Sakini chickens with Red Jungle Fowls from different countries in the natural ranges of Red Jungle Fowls (RJF) provides the first direct evidence that Red Jungle Fowl from India is the progenitor of the Nepalese Sakini chicken in the country as they share the same haplotype and/or lies in same haplogroup (**Figure 1b**).

It can be suggested that these birds have gone through multiple waves of domestication and contain many divergent matrilineal genetic components. **Figure 1a**, however, suggests that Nepalese Sakini chickens were more genetically close to *Gallus gallus gallus* in comparison to *Gallus gallus bankiva*. With the chickens, people intend to perform intense interbreeding between the breeds and sometimes even to carry out hybridization with wild jungle fowls in order to generate attractive flocks with unique phenotypes. The extensive gene flow among different breeds from the region and the introgression from jungle fowls could lead to this pattern.

Population genetic diversity of Nepalese Sakini chickens

The diversity indices calculated for Nepalese Sakini chickens showed a high haplotype diversity at 0.813 with a SD 0.065. The nucleotide diversity is more suitable parameter than haplotype diversity to estimate the genetic diversity within or between the populations. The nucleotide diversity (Pi) of Nepalese Sakini chickens is 0.00525 with a SD 0.00091 the average number of nucleotide differences per site among the 14 haplotypes. The high value in haplotype diversity infers the breeds to have richer gene pool [22]. As the undertaken study also has high Hd value, it can be deemed that the Sakini chicken breed has a richer gene pool. Nisar et al. (2019) revealed that the haplotype diversity of rural chicken population of Pakistan is high (0.825+-0.051); this higher Hd value is due to traditional and uncontrolled crossbreeding methods with different chicken breeds [23]. DLS (2020/21] reports that the number of poultry in rural areas outweighs urban areas and there has been an introduction of exotic commercial breeds in order to improve the productivity of the indigenous chickens [3]. As per our investigation, we found that the Nepalese farmers follow traditional and random breeding of backyard poultry which has resulted in a higher Hd value. Since this study reveals the higher genetic and haplotype diversity in Nepalese Sakini chickens, future research relating to the crossbreeding of the indigenous chickens with different commercial breeds of different countries could be carried out for pursuing the specific possible maternal lineages embodied by the unique Nepalese chicken breeds, e.g. the Sakini chickens.

Conclusion

This study has proved that mtDNA and more specifically D-loop HV 1 segment is a powerful molecular tool to understand the origin and genetic diversity of the specific breeds of domestic animals. The present study is the genetic characterization of Nepalese Sakini chickens for the first time in the country. The results show a high mtDNA D-loop diversity and indicate multiple maternal origins for Nepalese Sakini chickens. The high diversity in mtDNA haplotypes and haplogroups indicate extensive resources both for conservation and/or improvement by breeding. The present study has given the scientific basis for the claim that Red Jungle Fowl is the wild progenitor for Nepalese domestic chicken, Sakini. Our data based only on the Nepalese Sakini chickens of the central part of Nepal, further study

covering national level collection of samples from domestic chicken populations is required in order to understand the gene flow, especially for the haplogroup A. The information from our study, however, will serve the scientific basis for the development of rational policies supporting conservation efforts and provides directions for future research for developing sustainable genetic improvement approaches.

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