Mitochondrial DNA variation of indigenous goat populations from *Peste-des-petits-ruminants* outbreak in South Kivu, Democratic Republic of the Congo

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Abstract

This study was conducted to investigate the genetic diversity at *d-loop* of mitochondrial DNA and establish the possible maternal lineages of indigenous goat breeds in Democratic Republic of Congo (DRCongo). Phylogenetic relationships among and within 111 goats from three indigenous populations from a *peste-des-petits ruminants* outbreak in South Kivu, Eastern of Democratic Republic of the Congo and 22 goats sequences from the gene bank were analysed using mitochondrial control region sequences (*d-loop* region).

The results show that a total of 120 segregating sites, 56 haplotypes and 124 mutations were found in a 1220-bp sequence. The mean haplotype diversity and nucleotide diversity were 0.971±0.007 and 0.011±0.002, respectively with the overall number of nucleotide differences of 10.73. The phylogenetic analysis showed that all goat sequences were clustered into two haplogroups (A and B), of which haplogroup A was the commonest. The global analysis of molecular variance (AMOVA), incorporating all the three populations independent of any hierarchical clustering, indicated that 83.22% of the total genetic variation present in studied goats was explained by genetic differences between individuals (pv= 0.327), 11.18% among groups (pv=0.000*) and only 5.60% of the variation was attributed to genetic differences between populations (pv=0.003*). These results conclude that there are high levels of intrapopulation diversity in Mwenga-Shabunda, Fizi and Kalehe goats and the weak phylogeographic structuring, thus, suggested that there existed strong gene flow among goat populations probably caused by extensive trans-border movement of goats in the past.

Key words: *d-loop, genetic diversity, goat, haplotype*

Introduction

In Democratic Republic of the Congo, there are more than 21 million goats out of 350 million of goats found in Africa (FAOSTAT 2014). These goats are kept for milk and meat, mainly for family consumption, and inhabit a wide range of environments, extending from tropical to cool temperate climates. Previous studies have been classifying on molecular basis six mtDNA *d-loop* lineages [A, B (B1, B2), C, D, F, G] which have weak phylogeographic structure (Chen et al 2005; Naderi et al 2007). Indigenous goats (*Capra hircus*), when compared with their exotic counterparts, are better adapted to survive and reproduce under the region's harsh environmental conditions (Jimmy et al 2010).These indigenous goats also often possess valuable traits such as disease tolerance/resistance, high fertility, good maternal qualities and longevity, all of which are qualities that form the basis for low-input, sustainable agriculture (Bruford et al 1993).Goats play an important role in African culture as they are used for gifts, dowry and cultural rituals (Okomo-Adhiambo 2002).Some of their products like milk and bile are also medicinal (Bruford et al 1993).Moreover, there is a general concern that the genetic diversity within Africa's goats is disappearing through breed substitution, indiscriminate cross-breeding and the absence of breed development programmes (Naderi et al 2007).

Indigenous goat breeds are also not well characterized or have been scantily defined with very limited information available suggesting that most of the goat populations could disappear before they are identified.The mitochondrial DNA (mtDNA) polymorphism, especially the displacement loop (*d-loop*) region, has been largely applied to understand phylogenetical relationships in many animal species, including cattle (Mannen et al 2004), pig (Giuffra et al 2000), sheep (Hiendleder et al 2002), chicken (Liu et al 2004) and goat (Luikart et al 2001; Mannen et al 2001; Sultana et al 2003; Chen et al 2005).Based on former reports, Tarekegn et al (2016) summarized the global distribution and routes of divergence of indigenous goats and stated that the initial goats (i.e. variant B) arrived in the Canary Islands by the first settlers 3000YA. In the same year, the first inhabitants of the Canary Islands settled at the archipelago carrying a small number of domestic animals in 2200YA. On the other hand, the pair wise F_{ST} genetic distances plot indicates that the Canary goats are closer to goats from Middle East than the ones of North Africa suggesting the Canary goats diverged from the center of origin through Mediterranean Sea routes instead of terrestrial. Moreover Capote et al (2004) showed that the variant A found in some of the Canary Islands breeds might be due to the introgression between the native goats (variant B) with European and African breeds around 500-600YA following the colonization of Spanish. Peste-des-petits-ruminants (PPR) outbreaks were severally reported and confirmed in many neighboring countries to DRCongo such as Tanzania (Kgotlele et al 2014), Uganda (Bonny et al 2011) and Kenya (Simon et al 2015).The indigenous goats of South Kivu, DRCongo are still none identified and uncharacterized. Thus ,this study aimed at investigating the genetic diversity at *d-loop* of mitochondrial DNA and establish the possible maternal lineages of indigenous goat breeds in Democratic Republic of Congo.

Materials and methods

Sample collection and DNA extraction

A total of 111 blood and tissue samples were collected from farmers' flocks in three different agro-ecological areas, representing three indigenous uncharacterized goat populations of Democratic Republic of Congo which were confirmed previously with *Peste-des-petits-ruminants (PPR)* using both competitive Enzyme Linked Immunosorbent Assay (cELISA) and reverse transcriptase polymerase chain reaction (RT-PCR) tests. The sampling areas include Mwenga-Shabunda, Fizi and Kalehe in South Kivu region (Figure 1).

Blood samples were drawn out from the jugular vein with a volume of 9 ml under aseptic conditions using ethylene diamine tetra acetic acid (EDTA) anticoagulant tube (BD Biosciences, Franklin Lakes, USA). The collected samples were placed in icebox, taken to the laboratory and stored at -20 ^{0} C until when subjected to DNA extraction. Total genomic DNA was extracted from the samples using the DNeasy blood and tissues extraction kit (Qiagen, Hilden, Germany, Model: 69506) following manufacturer's procedure. The DNA quality and concentration were tested by Quibit (broad range and high sensitivity) bio analyzer, nanodrop; 1.0 - 1.5% agarose gel electrophoresis with the buffer TAE 0.5x was used to evaluate the degradation.

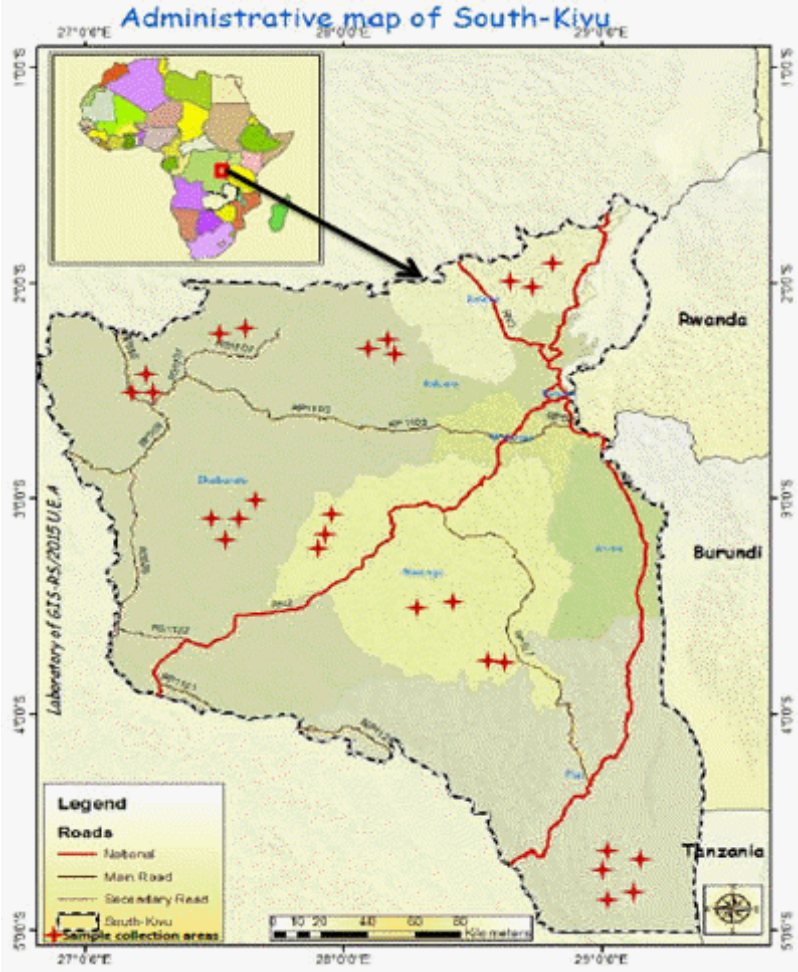


Figure 1. Geographical location of the sampling sites in stars

PCR amplification and sequencing

Conventional PCR amplification of mtDNA *d-loop*region was carried out. A pair of primers forward: 5'-GAAGCCATAGCCTCACTATC-3 and reverse primer: 5'-GTTGGTACACTCATCTAGGC-3') were used to amplified a 1220 base pairs fragment (Figure 2). PCR amplifications were performed using AccuPower® PCR Premix (Bioneer-Daejeon, Korea) to which 0.2 µM of each primer, 1.5% Hi-DiTM formamide (Applied Biosystems USA), 0.005mg of Bovine Serum Albumin (ThermoScientific), and 50 ng of template DNA were added in a final reaction volume of 20 µl. A one stage touch-down PCR cycling profile was used for PCR amplification. It involved an initial denaturation step at 95^{o}C for 1 min that

was followed by the first stage of amplification of thirty five cycles involving a denaturation step at 95^{o}C for 30 sec, annealing at 57^{o}C for 30sec, and extension at 72^{o}C for 60 sec. A final extension step at 72 ^{o}C for tenminutes completed the PCR. The PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden Germany) following the manufacturer's instructions. The purified products, 111 samples in total, were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Chemistry (AppliedBiosystems) and the ABI Prism 3130XL automatic capillary sequencer (Applied Biosystems, USA) following the manufacturers recommendations.

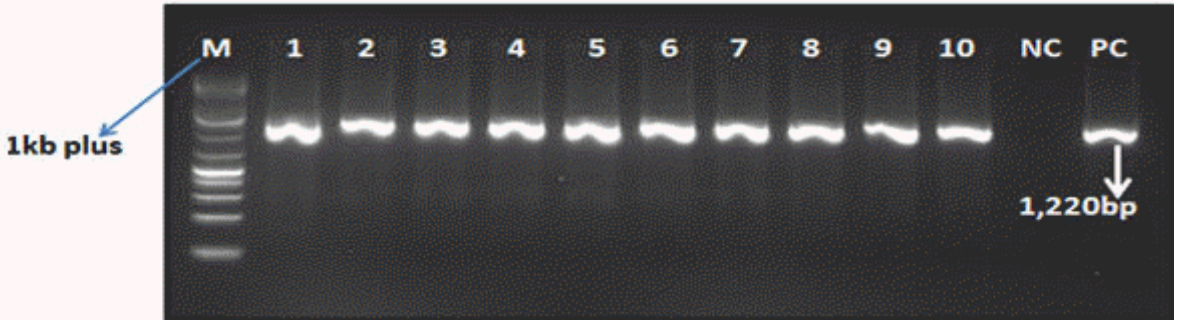


Figure 2. Electrophoresis Gel image of the mtDNA *D*-loop region amplified

Data analysis

All the chromatograms were generated and visualized with the CLC workbench 7.0.4 (CLC Bio-Qiagen). All the 111 mtDNA *d*-loop region sequences belonging to goats from Mwenga-Shabunda, Fizi and Kalehe regions of South Kivu in Democratic Republic of Congo were used in this analysis. Variable sites were scored/called against the *C.hircus* reference sequence GenBank accession number GU223571 (*direct submission*) that was retrieved from the GenBank database. To facilitate the recognition of haplogroup status of each individual, 22 goat mtDNA Hypervariable I region reference sequences (HV1: 481bp) belonging to the six known haplogroups/ lineages that were recommended by Naderi et al (2007) were also downloaded from GenBank and included in our analysis.

Multiple sequence alignments were done in CLC working bench employing the ClustalW algorithm and edited manually in MEGA 6 (Tamura et al 2013).The total of 111 sequences were generated and collapsed into haplotypes using the DnaSP package v5 10.01 (Librado et al 2009). The level of genetic diversity represented as the number of haplotypes, haplotype diversity (h) and its standards error (SE), nucleotide diversity (π) and its standards error (SE). The mean number of nucleotide differences between haplotypes was determined for each population using Arlequin 3.0. Phylogenetic tree was constructed for all the haplotypes using the Neighbour-Joining (NJ) algorithm following 1000 bootstrap replications with a Kimura 2-parameters model as implemented in MEGA6 to visualize the genetic relationship between individuals and populations. To complement the NJ tree while obtaining further insights, and in greater detail, into the genetic relationships between the haplotypes, the median-joining (MJ) network of haplotypes was constructed using the Network v4.6 software (http://www.fluxus-engineering.com/sharenet.htm).

Tajima's D and Fu's Fs tests were also conducted to determine whether patterns of mitochondrial sequence variation were consistent with predictions of the neutral model and to infer the demographic history of these three goat breeds as suggested by Santos et al (2010). The goodness of fit of the observed pattern to that expected under a demographic equilibrium was tested using the sum of squares deviation (SSD) of the goodness of fit statistic and the raggedness index as suggested by Harpending (1994). To evaluate the partitioning of genetic diversity and variation amongst populations and groups of populations, the analysis of molecular variation (AMOVA) was performed in Arlequin.

Results

Mitochondrial DNA sequence variation and genetic diversity

Out of 111 mtDNA *d-loop* sequences analysed, a total of 120 polymorphic or variable sites out of which 40 were for singleton variable sites and 80 for parsimony informative sites in a 1220-bp sequence and 56 haplotypes were observed. All the three populations were therefore defined by a high genetic diversity level. The total number of mutation recorded was 124. The haplotype diversity was very high in the studied goats with a value above 0.971 (Table 1).

The goat population of Shabunda-Mwenga and Kalehe showed the highest level of haplotype diversity (*H*d = 0.961 ± 0.010) and (*H*d=0.974 ± 0.019) respectively while the lowest level was observed in Fizi goats (*H*d = 0.873 ± 0.051). Similarly, the Table 1 shows that the average nucleotide diversity in the studied population was 0.011 ± 0.002 and ranged from 0.006± 0.001 in Fizi population to 0.010 ± 0.002 in Shabunda-Mwenga population which is closer to Kalehe goats (π =0.006± 0.001). The average number of nucleotide differences was 10.7 ranging from 6.75 for Fizi to 11.7 for Shabunda-Mwenga goats.

Table 1. Genetic diversity of mtDNA *d-loop* haplotypes of goats

Population/breed	N	S	Eta	H	Hd±SD	π ±SD	k
Shabunda-Mwenga	55	72	72	27	0.961 ± 0.010	0.010 ± 0.002	11.7
Kalehe	22	65	66	16	0.974 ± 0.019	0.009 ± 0.002	10.7
Fizi	32	42	42	16	0.873 ± 0.051	0.006± 0.001	6.75
Overall	**109**	**120**	**124**	**56**	**0.971 ± 0.007**	**0.011 ± 0.002**	**10.7**

Key: ***N*** *= Number of Individuals;* ***S*** *= segregating sites;* ***Eta*** *= No. of mutation;* ***H*** *= No. of Haplotypes;* ***Hd*** *= Haplotype diversity;* ***SD*** *= standard deviation;* $\boldsymbol{\pi}$ *= Nucleotide diversity and* ***k*** *= Average number of nucleotide differences.*

Population phylogenetic analysis

To construct the phylogenetic relationship using neighboring joining algorithm and phylogenetic network, we used only the HVI region. The DRC goats were aligned together with 22 reference sequences which represent the five globally identified haplogroups. The analysis shows that the

DRC goats were divided into two distinct mtDNA Lineages A and B (Figure 3 and 4). Lineage A had a total of 110 individuals (99.1%) and only one individual from Fizi goat population (0.9%) was aligned to lineage B. The Median-joining (MJ) network tree revealed the weak clusters to any population. Moreover, all the goat population from Shabunda-Mwenga and Kalehe clustered together in the haplogroup A (Figure 5).

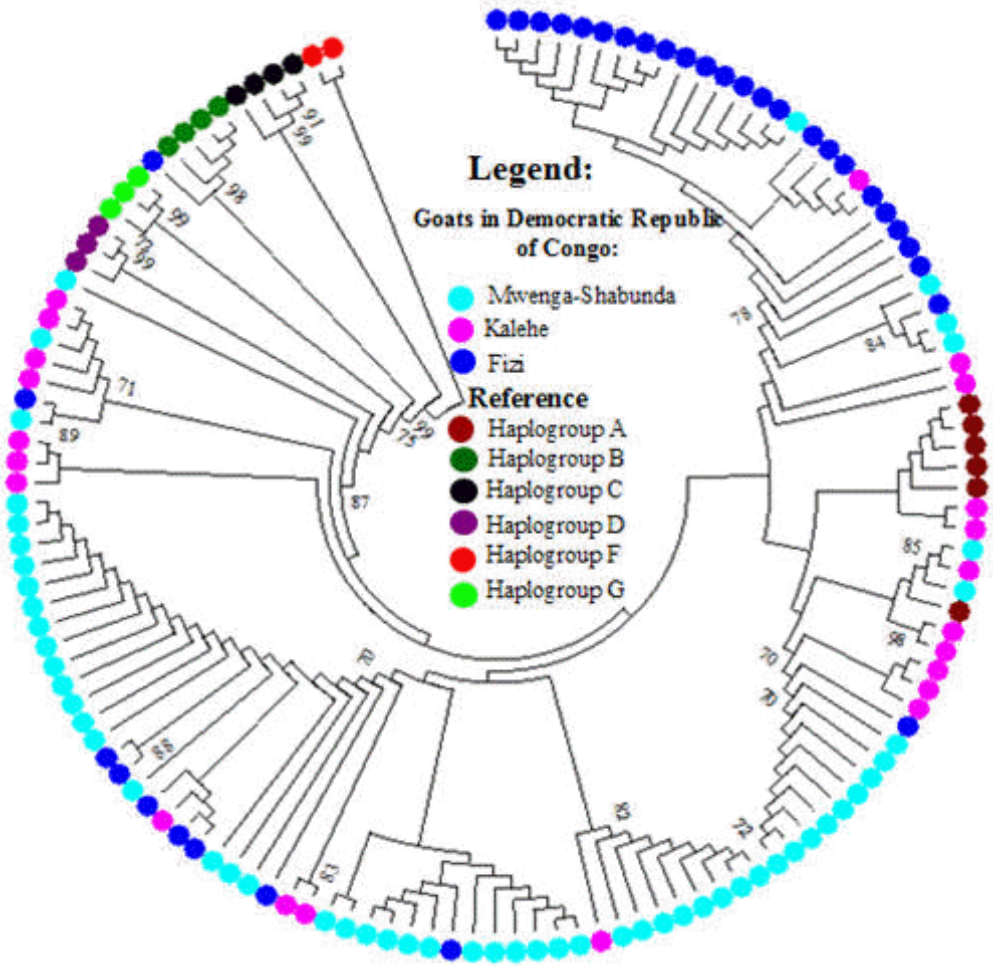


Figure 3. Neighbour joining tree of the three DRCongo goat populations, six reference haplogroups.

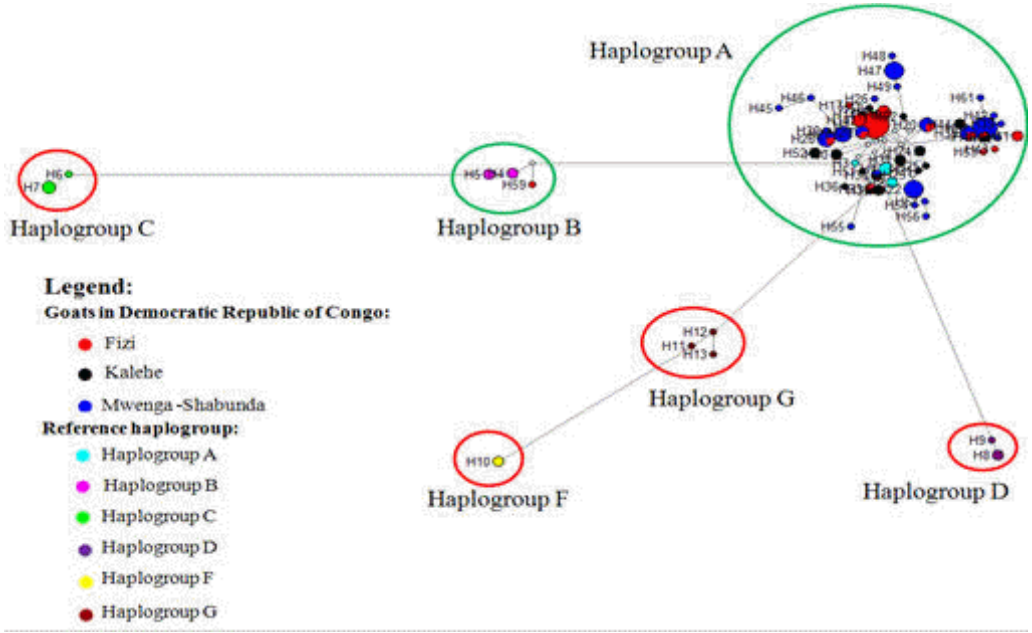
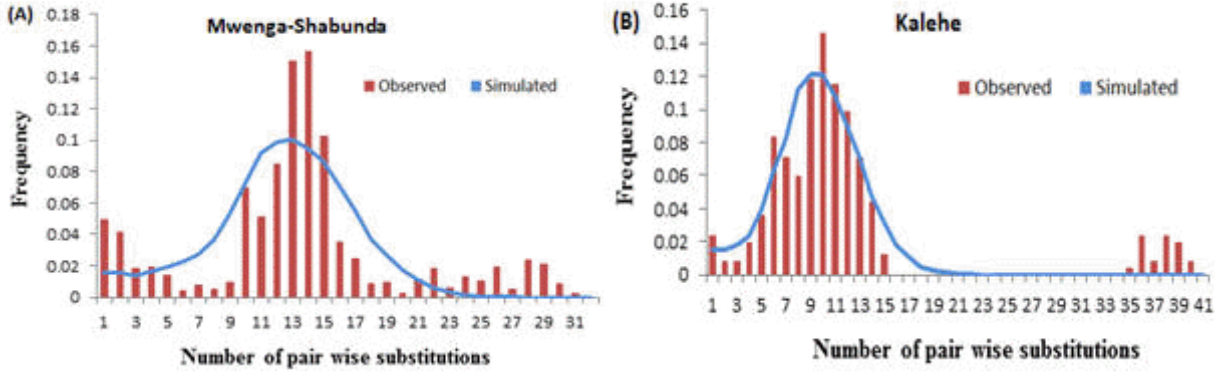


Figure 4. Median-joining network for the 56 mtDNA haplotypes of Shabunda-Mwenga, Kalehe and Fizi goats represented by red and yellow circles, respectively.



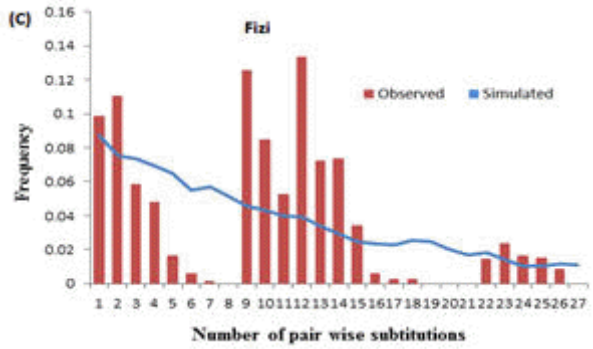


Figure 5. Mismatch distributions for mtDNA haplogroups of DRCongo indigenous goats in the respective haplogroups

Population differentiation

The result from AMOVA incorporating all the three populations independent of any hierarchical clustering indicated that 83.22% of the total genetic variation present in indigenous goats in Democratic Republic of Congo was explained by genetic differences between individuals within populations, 11.18 % (P=0.000) among groups and only 5.6% (P=0.003) among population within groups (Table 2).

Table 2. Analysis of Molecular Variance based on haplogroups and population groupings

Source of variation	d.f	Sum of squares (SSD)	Variance components	Percentage of variation	*p-value*
Among groups	1	50.875	0.720 Va	11.18	0.000
Among populations within groups	1	16.682	0.360 Vb	5.60	0.003
Within populations	108	567.920	5.358 Vc	83.22	0.327
Total	**110**	**635.477**	**6.438**	**-**	**-**

Key: SSD=Sum of square deviation, V=Variance components.

The pair-wise comparison values (F_{ST}) was 0.053 between Kalehe and Shabunda-Mwenga goats, 0.18573 between Fizi and Shabunda-Mwenga and 0.160 between Fizi and Kalehe (Table 3).

Table 3. Pairwise distance matrix (F_{ST}) of the goat populations studied

Population	Shabunda_Mwenga	Kalehe	Fizi
Shabunda_Mwenga	0.000	-	-
Kalehe	0.053	0.000	-
Fizi	0.186	0.160	0.000

Population dynamics

To assess population expansion events, mismatch distributions and Fu's *F*s statistic were used in this study and the results showed that each population was characterized by a bimodal mismatch distribution pattern as presented in Figure 4. For Shabunda-Mwenga and Kalehe goat populations, the observed pattern did not differ significantly from one expected for expansion, but it differs from the population of Fizi (Table 3).

These results were supported by both the Tajima's *D* and Fu's F_S statistics presented in Table 4 showing the presence of the population expansion.

Table 4. Population demographic and neutrality test in the DRCongo goat populations

Population	N	S	SDD	Raggedness index "*r*"	Tajima's *D* (p)	Fu's *FS* (p)
Shabunda_Mwenga	55	71	0.018	0.013	0.240	0.333
Kalehe	22	65	0.009	0.014	0.039	0.209
Fizi	32	42	0.013	0.031	0.066	0.322

*Key: N=Number of sequences; S=Segregating site; SSD=Sum of square deviation; ns=non-significant; *= significant at p<0.05; **=significant at p<0.01.*

Discussion

In this study, the control region of mtDNA (*d-loop*) was sequenced and analyzed to investigate the genetic diversity and origin of DRC indigenous goat populations. Two maternal haplogroups (haplogroup A and B) and high genetic diversity were detected from 56 haplotypes in a 1220-bp sequences of 111 sequenced animals and three goat populations studied that include Shabunda-Mwenga, Kalehe and Fizi. Haplotype B was reported in few populations of Namibia and South Africa goats (Chen et al 2005).The very high genetic diversity obtained in the current study could be partly explained by presence of high mutation rate in the control (*d -loop*) region as suggested by Naderi et al (2007). Several authors among them Luikart et al (2001) and Sultana et al (2003), Chen et al (2005), Naderi et al(2007) found multiple maternal haplogroups of domestic goat.

The findings of mtDNA haplotypes cluster in six haplogroups including A,B,C,D,F and G are supported with the MJ network analysis that we obtained from this study (Figure 4), with the predominance of haplogroup A. The few number of median vectors observed in these studies compare to literature gives clues that there could be animals which were not samples or extinct among Fizi goat's population. It was found globally a low phylogeographic structure among domestic goats (Naderi et al 2007).The median vectors found represent haplotype which were present in DRCongo could represent haplotypes that existed in the original *Caprine* gene pool but were not sampled or they were introduced but became extinct.

Haplogroup A generally had a higher level of genetic diversity compared to haplogroup B, a total of 10.7 nucleotide differences, 120 segregating sites and 124 mutations.Similar results were reported in three goat populations in Morocco, whereby 64 polymorphic sites were detected and 40 haplotypes belong to haplogroup A(Benjelloun et al 2015).Colli et al(2015) also reported 229 polymorphic sites in goat matrilineal variability in the whole mitochondrial genomes analysis. Moreover, 83.22% of the total genetic variation present in studied goats was explained by genetic differences between individuals within populations, 11.18% among groups and only 5.60% of the variation was attributable to genetic differences between populations (Table 2). This estimation is similarly to previous fundings where83% of the variationwithin population were observed in Indian goats,but differed from SCA goats where 69% of variation was observed within population and 78.7% for European, African and Asian goats compiled(FAOSTAT 2014). Morevoer 77% of goat genetic variation at global level that included 54 countries (Naderi et al 2007). The 11.1% and 5.6% of variations explained among breeds within geographic regions and among geographic regions indicating weak geographic structure concurrently because of the widest coverage of global distribution of haplogroup A.

The haplotype diversity of 0.971 obtained in the current study (Table 1) was low compare to the estimate of haplotype diversity for Iberian goats (0.996) and European goats (0.994). However, this found haplotype diversity in DRCongo goat's population is higher than estimates of Sicily goat'shaplotype diversity which range between 0.806-0.969, South and Central American (0.963) and 0.965 for Atlantic goat populations (Amills et al 2004; Amills et al 2008).Moreover the haplotype diversity estimate of about 0.988 was reported for goats lineages mtDNA sequences analysis (Naderi et al 2007).Subsequently in the same year Naderi et al (2007) observed a large number of variable sites of about 336in a 558 bp of alignment sequences of HVI region.

On the other hand, lowest estimates 0.873±0.051 of nucleotide diversity were observed in Kalehe population compared to the rest of DRCongo goats. Moreover Kalehe goat's population showed relatively higher differentiation than the rest of the studied goats. The phylogenetic network, F_{ST} and populations admixture analyses indicated in table 3, figure 4 and 5 support this observation. This could be explained by level of gene flow towards Kalehe area is minimal and/or the Kalehe goat habitat could be unfavorable for other DRC indigenous goat populations to adapt the local environment. Based on the information from mtDNA, it is noted that signatures of population expansion can be detected through frequency distribution of the number of pairwise differences between haplotypes and thus statistics based on the mismatch distribution.

The bimodal distributions observed in the graph of population expansion, in the current study, indicate there were one major and one minor events of population expansions in DRCongo goat populations in sometimes ago (Figure 5). Moreover, the positive and non-significant *F*s values obtained in our study confirm a slow populations' expansion (Table 4). The negative *F*s estimate (*F*s = -23.57<0.01) was observed for Chinese goats (Chen et al 2005).The human socio-cultural and economic interactions could explain the goats' demographic expansion. Moreover recently, the curves of multimodal mismatch were found in Anatolian Black and Angora populations (Akis et al 2014).

Conclusion

- The results indicate a very high mtDNA control region differentiation between Shabunda-Mwenga, Kalehe and Fizi indigenous goats of South Kivu province, DRCongo.

- The three goat's populations of South Kivu have undergone population expansion in the past, which reflects differences in their demographic histories.
- There is existence of two goat's haplotypes in South Kivu. Haplogroups A (99.1%) and B (0.9%).

Acknowledgements

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