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Assessing the genetic diversity of South-western Nigerian Indigenous Pig (*Sus scorfa*) using mitochondrial DNA D-loop sequence

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***Target audience:** Farmer, Geneticists, Animal Scientist, Academic researchers*

Abstract

In this study, the maternal genetic diversity and phylogenetic relationship of South-western pig population were assessed. Deoxyribonucleic acid (DNA) was extracted from air-dried blood spotted on Fast Technology for Analysis (FTA) card. The extracted DNA were amplified with predefined mitochondria (mtDNA) primers. A total of 843 base pair fragment of the mitochondrial DNA (mtDNA) D-loop region of 30 pigs were analyzed. Seven (7) haplotypes and 64 polymorphic sites were identified, with no insertion or deletion between nucleotide 3 and 835. The mean haplotypic and nucleotide diversity were found to be 0.381±0.058 and 0.315±0.155 respectively. The phylogeny revealed one divergent haplotype clade, suggesting one possible maternal lineage (European domestic pig) in South-west Nigerian pigs. The median joining network formed a star-like pattern, suggesting population expansion from a small number of founding ancestor (IFE1). Genetic variation within and between populations accounted for 63.32% and 36.68% of the total genetic variation respectively. This study concluded that there was relatively high genetic diversity in our indigenous pig population, thus, will probably pave way for preservation and improvement of Nigerian pigs as genetic resources.

***Keywords:** mtDNA, genetic variation, phylogenetic relationship*

Description of Problem

Pig domestication began about 9000–10 000 YBP at multiple sites across Eurasia, followed by their subsequent spread at a worldwide scale according to genetic and archaeological findings. Development of local breed throughout the centuries led to the foundation, mostly during the nineteenth century, of current modern breeds with defined phenotypes and production abilities (1). Overall, molecular studies investigating the pig breeds and populations in Nigeria seems non-existing. Most researches on pig are been overshadowed by the superficiality of the research work and the limited breeds and populations investigated (2). mtDNA sequence variation has been used extensively to study the genetic structure and matrilineal origin of farm animals (3, 4, 5, 6, 7). However, mtDNA reports about domestic pig are relatively sparse and especially in Nigeria.

The understanding of Nigerian pigs' genetic diversity and origin is very crucial for its characterization as an animal genetic resources (AnGR), to detect whether our pig populations are carriers of genetic variants which may be important for preservation and may be a source of new alleles that could be used for further

improvement of commercial pig breeds, thus, the use of molecular tools particularly mitochondria DNA (mtDNA) because it's maternally inherited in most species, highly polymorphic, high evolutionary rate and do not undergo recombination (8, 9, 10, 11).

In the present study, the sequences of the D-loop hypervariable 1 (HV1) segment of the mtDNA was used to study the genetic diversity and relationship of South-western Nigeria indigenous pig.

Materials and Methods

Blood collection and DNA extraction

Blood (2 ml each) was obtained from the ear vein of 30 unrelated individual pig from three towns (Ife, Ikirun and Ikire) in Osun State, South-western Nigeria using a new needle and syringe for each animal to avoid cross contamination, and dropped on the FTA classic cards (Whatman Bioscience, UK). Genomic DNA was extracted from air-dried blood spotted on FTA Classic cards stained as described in detailed previously by (7)

PCR amplification

The PCR was amplified from the D-loop region of mtDNA using L99 (5'-CCAAAGCTGAAATTCTAACTAAA-3') and H451 (5'-GGTGAGATGGCCCTG AAGTAAG-3') as forward and reverse primer respectively (12). All polymerase chain reactions were performed in a 30µl reaction volume containing 5µl of genomic DNA, 25 µl of PCR mix (1 µl of 5mMdNTP, 1 µl of 10Mm of each primer, 2.5 µl of 25mM of $MgCl_2$, 2.5 µl of 10x PCR buffer, 16.8 µl of Nuclease free H_2O and 50.2 µl of 10U/ µL of Surf Hot *Taq* DNA) (Stabvida, Spain). All PCR amplifications were carried out on Agilent Surecycler 8800 thermal cycler (Stabvida, Spain). PCR conditions were: Initial denaturation at 96 ^oC for 15 minutes, followed by 35 cycles of denaturation at 94 ^oC for 30 seconds, annealing at 55 ^oC for 45 seconds and extension at 72 ^oC for 1 minute for extension, and final extension step at 72 ^oC for 5 minutes.

Sequencing of D-loop fragments

A total of 25µl comprising 20 ng of purified DNA and 25 µl of PCR mix (1 µl of 5mMdNTP, 1 µl of 10Mm of each primer, 2.5 µl of 25mM of $MgCl_2$, 2.5 µl of 10x PCR buffer, 16.8 µl of Nuclease free H_2O and 50.2 µl of 10U/ µL of Surf Hot *Taq* DNA) (Stabvida, Spain) was used for direct sequencing of HV-1 segment of the D-loop region on a BDTv3.1 (Applied Biosystems, USA) technology. The reaction mixtures were then transferred to a 96-well reaction plates of ABI 3730 XL Capillary DNA Analyzer (Applied Biosystems, USA), and the cycling parameters were: 96 ^oC for 1 minute for initial denaturation, followed by 30 cycles at 96 ^oC for 10 seconds of denaturation, annealing at 50 ^oC for 6 seconds, 60 ^oC for 4 minutes of extension, and final extension step at 12 ^oC for 10 minutes.

Data analysis

A total of 843bp including the hypervariable region 1 (HV1) was subsequently used for analysis. Viewing and editing of the sequences was done using Chromaspro. The consensus sequence was aligned against the reference sequence (GenBank accession number: NC_008830) excluding all gap using ClustalW implemented in MEGA 6.06 (13). Sequence variation indexes (number of haplotypes, haplotype diversity, nucleotide diversity) were calculated using DnaSP version 5 (14). A Neighbour-Joining (NJ) tree was constructed for identified pig haplotypes and reference haplotypes (GenBank accession number: EF545592, AY884732, AY884765, AB015094) with a 1000 bootstrap replicates using MEGA version 6.06 (13). A Median Joining (MJ) network analysis was constructed using NETWORK 4.6.1.2 (15). Genetic differentiation was

quantified using analysis of molecular variance (AMOVA), and test of selective neutrality (Tajima's *D* and Fu's *Fs*) were calculated using Arlequin 3.5.1.3 software (16).

Result

Sequence variation in mtDNA

The analysis of the mtDNA D-loop sequences of 30 sampled pigs (Table 1) showed 64 polymorphic sites, representing 1.89% of the total DNA sequence analysed (843bp) with no either insertion or deletion, in comparison with Warthog sequence (GenBank accession number: NC_008830). The pattern revealed high variations between nucleotide 3 and 835, and 7 haplotypes was found.

The sampled population shared a common haplotype (IFE1) having the highest frequency. The identified haplotypes, observed nucleotide polymorphisms in the D-loop HV-1 segment of the sampled pig sequences and frequencies.

Table 1: ***Observed nucleotide polymorphism in South-western Nigerian pig population***

	000000000000000000000011111111112222223333333444555555555566668	
	001122333344456678889913588889992377880122339179011556677701222	**N**
	387859028924640404593620012891252956013913480781001463534687025	
Ref	CTAAACTGAATTATTCCTATTACCTCAAACATTTAGTTTTATTATGTGTCGTTCTACCAGGGCC	
IFE1	GC..G.CATTC.GACTTCG....T.ATC.ATCCCG.CCA.TGAGCA.ACAAACTCCATGCAA..	24
IK13	GC..G.CATTCGGACTTCG....T.ATC.ATCCCG.CCA.TGAGCA.ACAAACTCCATGCAAAA	1
IK17	GC..G.CATTC.GACTTCG...AT.ATC.ATCCCG.CCA.TGAGCA.ACAAACTCCATGCAA..	1
KRE5	GCCCGACATTC.GACTTCGC.C.TGATCGATCCCCACCA.TGAGCAAACAAACTCCATGCAA..	1
KRE12	GCCCGACATTC.GACTTCGC.C.TGATCGATCCCCACCA.TGAGCAAACAAACTCCATGCAA..	1
KRE13	GC..G.CATTC.GACTTCG.A..T.ATC.ATCCCG.CCA.TGAGCA.ACAAACTCCATGCAA.A	1
KRE17	GC..G.CATTC.GACTTCG....T.ATC.ATCCCG.CCAATGAGCAAACAAACTCCATGCAA..	1

Vertically oriented numbers indicate the position of the variable site. Dots (.) indicate identity with the reference (Reference) sequence (GenBank accession number: NC_008830), while different base letters denote substitution. N: Number of individuals in each haplotype

Molecular diversity indices

The sampled pig populations have a haplotype diversity, nucleotide diversity and mean number of pairwise difference value of 0.366±0.112, 0.073±0.053 and 1.248±0.812 respectively Table 2). A total of 16 nucleotide substitution (13 transversions and 3 transition) and 16 polymorphic sites (14 singletons and 2 parsimony information sites) was observed.

The theta value based on expected homozygosity (Θ_H), based on average number of pairwise difference (Θ_π), based on number of alleles (Θ_k) and based on number of segregating site (Θ_S) were found to be 0.429±0.210, 1.248±0.903, 2.558±1.073 and 4.039±1.563 respectively.

It was observed that the population has more of thymine (36.04%) and less of guanine (12.71%).

Table 2: ***Sample size and population genetic diversity measure for South-western Nigerian pig population***

Parameters	Overall
Sample size	30
Haplotypes	7
Haplotypic diversity	0.366±0.112
Nucleotide diversity	0.073±0.053
Mean pairwise differences	1.248±0.812
Number of observed transitions	3
Number of observed transversions	13
Number of substitutions	16
Number of polymorphic sites	16
Number of observed indels	0
Θ_H	0.429±0.210
Θ_S	4.039±1.563
Θ_K	2.558±1.073
Θ_π	1.248±0.903
Nucleotide composition (%)	
C	24.79
T	36.04
A	26.46
G	12.71

θ_H: Theta value based on expected homozygosity; θ_k: Theta value based on number of alleles; θ_s: Theta value based on number of segregating sites; θ_π: Theta value based on the average number of pairwise differences; C: Cytosine; T: Thymine; A: Adenine; G: Guanine

Mismatch distribution and Neutrality test

The results of the further analysis carried out to test for population expansion (Table 3) showed a mismatch observed mean and variance of 1.248 and 6.763 respectively for the sampled pig population. The time of expansion (T) of 3.000 was observed in the pig population, with mutation parameters (θ_1 and θ_0) of 0.000 and 0.000 respectively.

A significant negative (p<0.01) Tajima's *D* and Fu's *Fs* value of -2.347 and -1.966 respectively were observed.

Table 3: ***Demographic expansion indices of South-western Nigerian pig population***

	Overall
Mismatch observed mean	1.248
Mismatch observed variance	6.763
T	3.000
θ_0	0.000
θ_1	0.000
D	-2.347**
P (Sim. D < Obs. D)	0.000
Fs	-1.966**
P (Sim. Fs < Obs. Fs)	0.089

T: time of expansion; θ_0 and θ_1: mutation parameters; D: Tajima's neutrality test; Fs: Fu's neutrality test.
**p<0.01

Network profiling and phylogenetic analysis

The Neighbor Joining dendrogram revealed that the identified haplotypes in this studied pig population clustered with European domestic pigs (Figure 1). This indicates a very close relationship between the sampled pig and domestic pig of European origin.

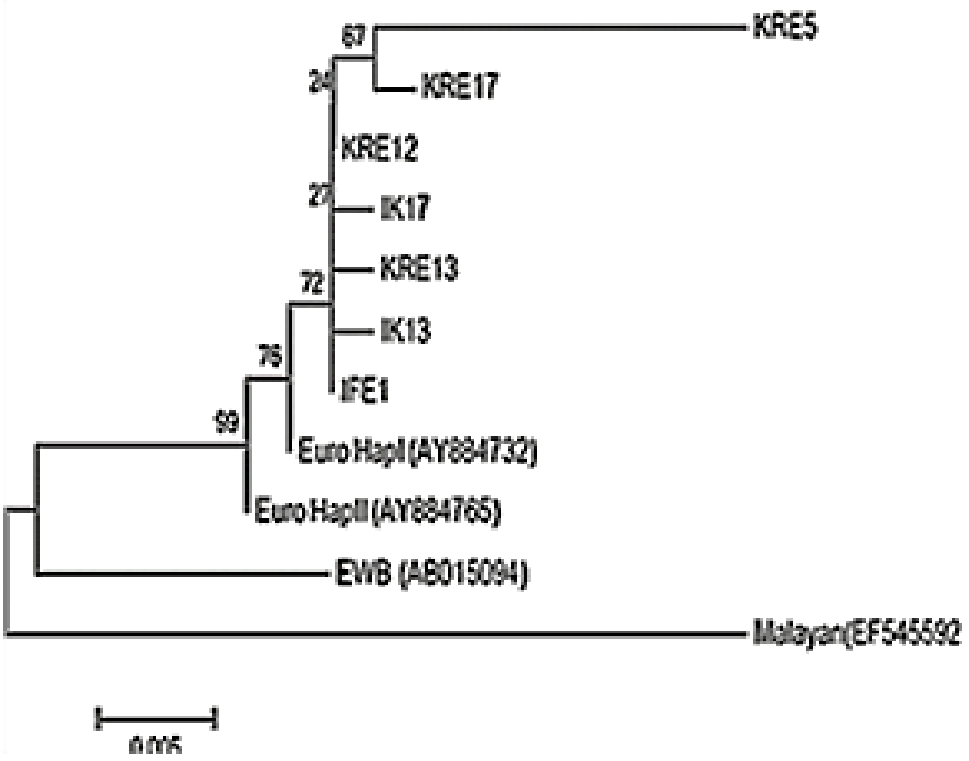

Figure 1: ***Neighbor-joining tree for 7 South-western Nigerian and other pig haplotypes.***

Neighbor-joining tree reconstructed for 7 South-western pig haplotypes, two European domesticated pig haplotypes (Euro HapI and HapII), one European wild boar (EWB) and Malayan wild boar (Malayan) using MEGA 6.06 software. The numbers at the node represent the percentage bootstraps values for interior branches after 1000 replications.

A median joining network of the sample pig population was constructed using all 7 haplotypes to better visualize the relationship among them (Figure 2). The network formed a star-like pattern and the topological structure was basically identical to the NJ tree, thus confirms the close relationship between sample pig population as well as illustrated that observed haplotypes belongs to a single expansion event centered on IFE1.

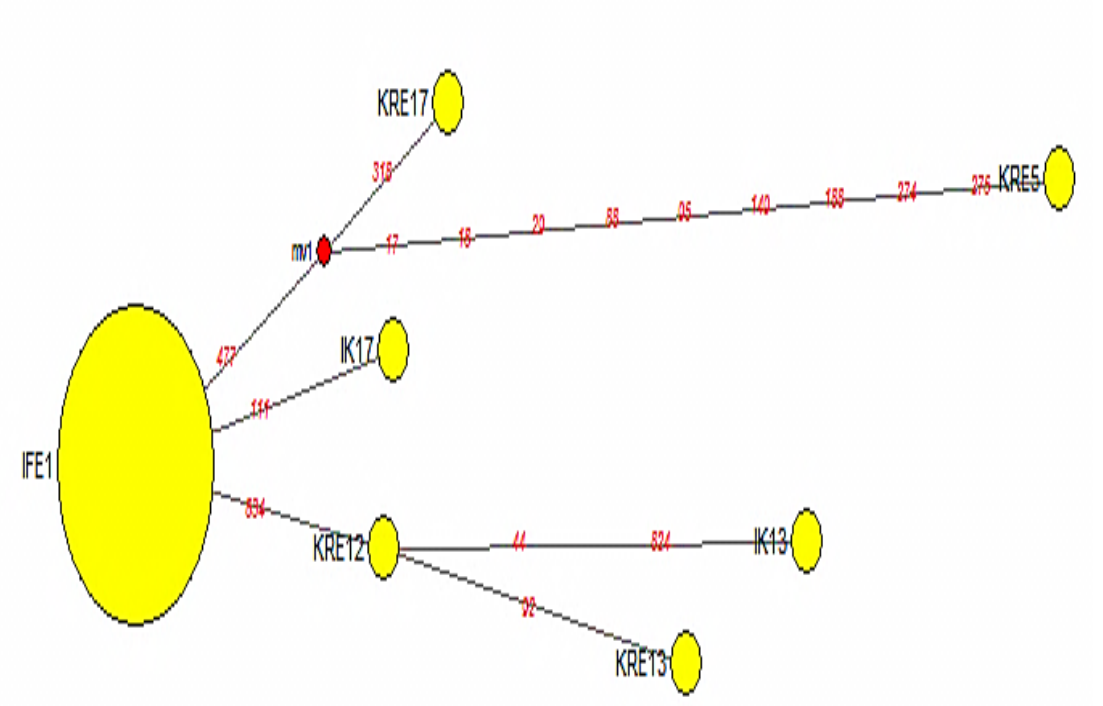

Figure 2: ***Median-joining tree for 7 South-western Nigerian***
Median joining network (ε=0) of South-western Nigerian pig haplotypes based on the polymorphic sites of the mitochondrial D-loop HV-1 region. Area of each circle is proportional to the frequency of the corresponding haplotype. Different classes of haplotypes are distinguished by use of colour codes (yellow=South-west). The red colour between the haplotype nodes refer to the positions of median vector

Maternal genetic differentiation

The analysis of molecular variance (Table 4) shows the variation in percentage of maternal genetic differentiation within and between sampled pigs, using Tajima-Nei distance method (17) and calculations were performed based on 1000 permutations. The genetic variation within population was 95.20% than the genetic differentiation among the populations was 4.80%, with a fixation index (F_{ST}) value of 0.048.

Table 4: ***Analysis of molecular variance***

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation	F_{ST}
Among populations	1	1.038	0.03075	4.80	0.04804
Within Populations	28	17.062	0.60936	95.20	
Total	29	18.100	0.64011		

F_{ST}: fixation index

Discussion

The polymorphism result indicated that mtDNA D-loop region is highly variable revealing a total of 7 haplotypes between nucleotide 3 to 835. The observed nucleotide variability was detected from a total of 64 polymorphic sites between sampled pigs and reference sequence (GenBank accession number: NC_008830). The high observed within population variation (95.20%) represents the phenotypic variation among individuals in the population, thus, indicating a panmictic population. The observed low haplotype number (7) may be due to low sample size and homogeneity status of the sampled population.

Haplotype diversity (Hd) and nucleotide diversity (Pi) of populations were main indexes for evaluating the mtDNA variation and genetic diversity of breed or population (18). The haplotypic diversity of sampled pigs was found to be 0. 366, and is lower when compared to the report of (12) on Hezuo pig in Gansu, China (0.963), Diqing pig in Yunnan, China (0.930), Aba pig in Sicuan, China (0.830) and Zang pig in Tibet, China (0.571) while the nucleotide diversity observed in this study (0.073) is higher than what was reported by (12) value of 0.0062 for Tibetan pigs. In general, average nucleotide difference (K) was higher compared to nucleotide diversity, thus, indicating a high genetic diversity.

The observed significant negative Tajima's *D* indicated that the sample pig population departed from equilibrium which may be as a result of past/recent population expansion, bottleneck effect or heterogeneity of mutation rates as opined by (19). However, observed negative Fu's *F* value provides a strong evidence of population expansion, which may be due to genetic hitchhiking, background selection and evolutionary force to drive a population expansion signature (20, 21).

The mtDNA phylogeny and network analysis revealed one divergent haplotype group as they clustered under domestic pig of Europe origin. This suggested that South-western Nigerian indigenous pig originated from one maternal lineage; the European domestic pig. The relatively high bootstrap values of NJ tree indicated a clear genetic sub-structuring between observed haplotype which could be attributed to relatively low non-random genetic intermixing between haplotypes.

The hierarchical analysis of molecular variance (AMOVA) indicated that 4.80% of maternal genetic differentiation resulted from variation among populations while 95.20% was due to contribution by genetic divergence among individual within population. This observed result indicated that individual pig still retains its genetic uniqueness and can be selected for breeding purpose.

Conclusion and Application

This study revealed that

1. The sampled pig population has a relatively high genetic diversity and low genetic differentiation, which could be utilized for breeding and conservation programme.
2. South-western Nigerian pigs has one ancestral maternal origin (IFE1), believed to be relative of European domestic pig.
3. mtDNA D-loop sequence is useful and highly informative for studying genetic diversity and variability, thus, should be employed in the identification of quantitative trait loci (QTL) for marker-assisted selection in pig genetic improvement as well as formulation of effective conservation strategies.

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