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Assessing genetic diversity and phylogeographic structure of duck *(Anas platyrhynchos)* in Nigeria using mitochondrial DNA D-loop sequences

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Abstract

In this study, the maternal genetic diversity and phylogenetic relationship of Nigerian duck populations were assessed. A total of 591 base pair fragment of the mitochondrial DNA (mtDNA) D-loop region of 87 indigenous ducks from two populations in Nigeria were analyzed. Seven haplotypes and 70 polymorphic sites were identified. The mean haplotypic and nucleotide diversity were found to be 0.381±0.058 and 0.315±0.155 respectively. The phylogeny revealed two divergent haplotype clades, suggesting two possible maternal lineage in Nigerian duck population, with the most commonly shared haplotype belonging to Mallard ducks (*Anas platyrhynchos*). 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 and differentiation, thus, this information will probably pave way for further evaluation studies, preservation and improvement of Nigerian ducks as genetic resources.

Keywords: Genetic Diversity, MtDNA, Nigerian duck, phylogeny

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Introduction

Duck is believed to be one of the earliest domesticated fowls in the world (CASS, 1979; Li et. al., 2010), and known to have resulted from Mallard (Wild duck) with South-east Asia as its center of origin (Wojcik and Smalec, 2007). Duck is one of the indigenous poultry species in Nigeria and its production is at rudimentary stage. Nigerian duck have been phenotypically characterized based on morphological characters which have provided a reasonable representation of their genetic difference, however, the accuracy of phenotypic characterization is often affected by the influence of the environment and the underlying genetic complexity (Yakubu et. al., 2011).

Overall, molecular studies investigating the duck breeds and populations in Nigeria seems non-existing. Most researches on duck are been overshadowed by the superficiality of the research work and the limited breeds and populations investigated (He et. al., 2008). The mtDNA sequence variation has been used extensively to study the genetic structure and maternal origin of farm animals (Olowofeso et. al., 2005; Adebambo et. al., 2009; Adebambo et. al., 2010; Ajibike et. al., 2015; Ajibike et. al., 2016). However, mtDNA reports about domestic duck are relatively scarce and especially in Nigeria.

The understanding of Nigerian indigenous ducks' genetic diversity and origin is very crucial for its characterization as an animal genetic resources (AnGR), thus, the use of molecular tools particularly mitochondria DNA (mtDNA) because it is maternally inherited in most species, highly polymorphic, high

evolutionary rate and do not undergo recombination. These features mean that each molecule as a whole usually has a single genealogical history through maternal lineage (Adebambo et. al., 2009).

In the present study, the sequences of the D-loop hypervariable 1 (HV1) segment of the mtDNA were used to study the genetic diversity and relationship of Nigeria duck populations.

Materials & Methods

Sample Collection and DNA extraction

A volume of 2 ml of blood was collected from the wing vein of 87 Nigerian indigenous ducks using new needle and syringe for each animal to avoid cross contamination between samples. The blood collected was dropped on FTA classic cards (Whatman® Bioscience, UK), allowed to dry and stored at room temperature. These cards were transferred into bags containing silica gel and transported to the laboratory for further analysis.

Genomic DNA was extracted from air dried blood preserved on FTA classic cards (Whatman Biosciences), using the recommended manufacturer protocol. The samples include 47 ducks from the Northern region of Nigeria representing population I (North) and 40 ducks from the Southern region representing population II (South).

Polymerase Chain Reaction (PCR)

Mitochondrial DNA amplification from the D-loop region of the duck mitochondrial genome was performed by using DL-AnasPF_L56 (5' – GTTGCGGGGTTATTTGGTTA - 3') as forward primer (Purwantini et al., 2013) and DL-AnasPR_H733 (5' – CCATATACGCCAACCGTCTC - 3') as reverse primer (Purwantini et al., 2013). 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, Portugal). All PCR amplifications were carried out on Agilent Surecycler 8800 thermal cycler (Stabvida, Portugal).

PCR conditions were: Initial denaturation at 96°C for 15 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 minute for extension, and final extension step at 72°C for 5 minutes.

Sequencing of D-loop fragments

Purified fragments with a DNA concentration of 30 ng/µl, was sequenced to determine the precise nucleotide sequence. Direct sequencing of HV-1 segment of the D-loop region was performed using DL-AnasPF_L56 primer (5' – GTTGCGGGGTTATTTGGTTA - 3'). Sequencing was performed with BDTv3.1 (Applied Biosystems, USA) technology and running was made in an ABI 3730 XL Capillary DNA Analyzer (Applied Biosystems, USA). A total volume of 25µl comprising 20 ng of purified PCR product as template 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).

The reaction mixtures were then transferred to 96-well reaction plates of ABI 3730 XL Capillary DNA Analyzer (Applied Biosystems, USA). The cycling parameters were: Initial denaturation was at 96°C for 1 minute, followed by 30 cycles of denaturation at 96°C for 10 seconds, annealing at 50oC for 6 seconds and extension at 60°C for 4 minutes, and final extension step at 12°C for 10 minutes. After the last cycle, there was a rapid thermal ramp to 4°C and holding until the purification of the sequence product was done.

Data analysis

A 591 bp long fragment, including the hypervariable region 1 (HV1), was subsequently used for analysis. Viewing and editing of the sequences was done using BioEdit software (Hall, 1999). The consensus sequence was aligned against the reference sequence (GenBank accession number: EU755254) excluding all gap using ClustalW in MEGA 6.06 (Tamura et. al., 2013). Sequence variation (number of haplotypes, haplotype diversity, nucleotide diversity, average number of nucleotide difference and average number of nucleotide substitutions per site between populations) were calculated using DnaSP version 5 (Librado and Rozas, 2009).

A Neighbour-Joining (NJ) tree was constructed for identified Nigerian duck haplotypes and reference lineage haplotypes (GenBank accession number: L16769, JN811041 and AY506945 - AY506948) with a 1000 bootstrap replicates using MEGA version 6.06 (Tamura et. al., 2013). A Median Joining (MJ) network analysis was constructed using NETWORK 4.6.1.2 (Bandelt et. al., 1999). Maternal genetic differentiation was further quantified using hierarchical analysis of molecular variance (AMOVA), mismatch distributions, Tajima's *D* and Fu's *Fs* were calculated using Arlequin 3.5.1.3 software (Excoffier et. al., 2005).

Results

Nucleotide polymorphism

The pattern of 591 bp mtDNA variability revealed a high variation between nucleotide 10 and

578 with North duck population having the highest polymorphism (67) and South duck has the lowest polymorphism (4). The two duck populations shared a common haplotype (IWO3), while North duck population has the highest unique haplotype of 5 and South duck population has only one unique haplotype. A total of 7 haplotypes (Table 1) defined by 70 polymorphic sites with no insertion or deletions from the 87 sampled Nigerian indigenous duck were observed. The identified haplotypes, observed nucleotide polymorphisms in the D-loop HV-1 segment of the Nigerian duck sequences and frequencies are shown in Table 1.

Table 1: Observed nucleotide polymorphism in Nigerian duck population

	000000000000000000000000000001111111111111111111111111112222244445555 **112344444555566667888888999990000001111123333345567777789125773455037 7** **092402579068915795016789135790456781678902678922573678974259120267106 8**	**N**	
		North	South
Ref	AACAACGCAACAACTAGCCCAGCACTCACAAAGCATTCCAGGACGCACTACCTTACTACTCGTTCGCAAG		
IWO3	..TCCAA.GT..CACCATTATATCACTTTC.CAGC.CTGCAACATA.TCGTTAGCACGTCTAACGATGGA	28	39
IWO43	TTTCCTA.GT..CACCATTATATCACTTTC.CAGCACTGCAACATA.TCGTTAGCACGTCTAACGATGGA	-	1
YA14	.....T.CACC.....G.....G.....	1	-
OG29	.....G.....G.....	15	-
OG35	.....A.....G.....G.....	1	-
OG43	.....C.....G.....G.....	1	-
GWA7	.....T...C....A.....G.....G.....	1	-

Vertically oriented numbers indicate the position of the variable site. Dots (.) indicate identity with the reference (Reference) sequence (GenBank accession number: EU755254), while different base letters denote substitution. N: Number of individuals in each haplotype

Molecular diversity indices

The results of molecular diversity indices of the sampled Nigerian indigenous duck (Table 2) revealed that North duck population has the highest haplotype (gene) diversity, nucleotide diversity and a mean number of pairwise difference value of 0.553±0.055, 0.448±0.221 and 31.784±14.119 respectively compared to the South duck population with 0.050±0.047, 0.003±0.004 and 0.200±0.254, while the overall haplotype (gene) diversity, nucleotide diversity and a mean number of pairwise difference value are 0.381±0.058, 0.315±0.155 and 22.326±9.929 respectively.

The sum of square frequency value was high in South duck population (0.951) and least in North duck population (0.459) while mean for all sampled populations was 0.624. A total of 68 nucleotide substitutions (38 transition and 30 transversions) and 4 (0 transition and 4 transversions) were observed in North and South duck populations respectively, with no either insertion or deletion. The North duck has the highest cytosine and guanine percentage of 31.49 and 14.56 respectively while the highest adenine (32.79) and thymine (25.79) percentage was observed in South duck population. The overall population has more of adenine and cytosine percentage of 32.48 and 30.15 respectively.

Table 2: Molecular diversity indices of Nigerian duck population

Parameters	North	South	Overall
Sample size	47	40	87
Haplotypes	6	2	7
Haplotypic diversity	0.553±0.055	0.050±0.047	0.381±0.058
Nucleotide diversity	0.448±0.221	0.003±0.004	0.315±0.155
Sum of square frequencies	0.459	0.951	0.624
Mean pairwise differences	31.784±14.119	0.200±0.254	22.326±9.929
Number of observed transitions	38	0	39
Number of observed transversions	30	4	33
Number of substitutions	68	4	72
Number of polymorphic sites	67	4	70
Number of observed indels	0	0	0
Θ_H	0.927±0.210	0.0394±0.0389	0.458±0.113
Θ_S	15.170±4.566	0.940±0.525	13.896±3.778
Θ_K	1.602±0.653	0.256±0.060	1.612±0.711
Θ_π	31.784±15.672	0.940±0.525	22.326±11.000
Nucleotide composition (%)			
C	31.49	28.57	30.15
T	21.73	25.79	23.60
A	32.22	32.79	32.48
G	14.56	12.86	13.78

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

The results of the further analysis carried out to test for population expansion (Table 3) using Tajima's (*D*) and Fu's (F_s) neutrality test for the sampled Nigerian duck population revealed that North duck population has the highest mismatch observed mean and observed variance of 31.784 and 499.955 respectively, while South duck population has 0.200 and 210.507, with an overall mismatch observed mean of 22.326 and 930.600 respectively. The time of expansion (T) of 3.000 was observed in North duck population while the South duck population shows no time of expansion, with both having mutation parameters (θ_1 and θ_0) of 0.000 and 0.000 respectively. A non-significant positive (P>0.10) Tajima's *D* value of 3.868 was observed in North duck population while a non-significant negative (P>0.10) value of -1.880 was observed for South duck population. A Fu's *Fs* value of 34.100 and 0.242 was observed for North and South duck population respectively, with an overall value of 34.434.

Table 3: Mismatch distribution of Nigerian duck population

	North	South	Overall
Mismatch observed mean	31.784	0.200	22.326
Mismatch observed variance	499.955	210.507	930.600
T	3.000	0.000	3.000
θ_0	0.000	0.377	0.000
θ_1	0.000	3414.017	0.000
D	3.868	-1.880	1.995
P (Sim. D < Obs. D)	1.000	0.004	0.931
Fs	34.100	0.242	34.434
P (Sim. Fs < Obs. Fs)	1.000	0.296	1.000

T: time of expansion;θ_0 and θ_1: mutation parameters; D: Tajima's neutrality test; Fs: Fu's neutrality test

Phylogenetic and Network analysis

The result of the Neighbour-joining analysis based on Tajima and Nei (1984) model using bootstrap method (1000 replications) revealed that identified Nigerian duck haplotypes were clearly differentiated into two distinct groups; with two haplotypes (IWO3 and IWO43) seems more closely related to the domestic duck (*Anas platyrhynchos* and *Anas zonorhyncha*) while five (OG29, OG35, OG43, YA14 and GWA7) haplotypes seems more closely related to the Muscovy breed (*Cairina moschata*) (Figure 1).The haplotype network analysis (Figure 2) further confirms the differentiation between the sampled Nigerian duck haplotypes, but, it clearly illustrates that Nigerian duck haplotypes belongs to a single expansion event centered on IWO3.

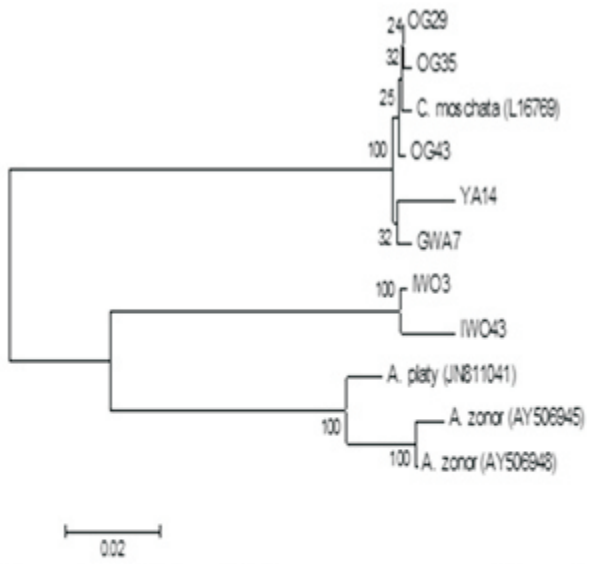

Figure 1: Neighbor-joining tree reconstructed from the 7 haplotypes identified in the 87 Nigerian duck sequences, 1 haplotype of *Cairina moschata*, 2 haplotypes of *Anas zonorhyncha* and 1 haplotype of *Anas platyrhynchos domesticus*. The percentage bootstrap value is represented by the numbers at the node after 1000 replication

Figure 2: Median-joining network (ε = 0) of Nigerian duck haplotypes based on the polymorphic sites of the mitochondrial D-loop HV1 region. Area of each circle is proportional to the frequency of the corresponding haplotype. Different classes of haplotypes are distinguished by the use of colour codes (yellow =North Nigerian and green South Nigerian duck). The red colour between the haplotype nodes refers to the positions of the median vector

Analysis of molecular variance

The analysis of molecular variance (Table 4) shows the variation in percentage of maternal genetic differentiation within and between Nigerian duck population based on sample locations. The calculations were performed based on 1000 permutations. The result revealed that there was a higher genetic variation within populations (63.32%) than among populations (36.68%), with a fixation index (F_{ST}) value of 0.367.

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	225.113	5.00868 Va	36.68	0.367
Within Populations	85	734.921	8.64613 Vb	63.32	
Total	86	960.034	13.65481		

FST: fixation index

Discussion

In this study, we investigated the genetic diversity and phylogenetic structure of Nigerian duck populations. The polymorphism result indicated that mtDNA D-loop region is highly variable revealing a total of 7 haplotypes between nucleotide 10 to 578. The observed nucleotide variability was detected from a total of 70 polymorphic sites between Nigerian duck populations and an ancestral duck sequence (GenBank accession number: EU755254). The observed within and between populations' sequence variation represents the phenotypic variation among individuals in the population. The low observed haplotype number (7) may be due to low sample size and homogeneity status of the sampled population, while the high rate of mtDNA D-loop variation may be attributed to migration and exploratory activities of human within the country.

Haplotype diversity (Hd) and nucleotide diversity (Pi) of populations were main indexes for evaluating the mtDNA variation and genetic diversity of breed or population (Li et. al., 2010). The average haplotypic diversity of sampled Nigerian duck populations was found to be 0.381, and is lower when compared to the intercontinental duck (0.82 to 0.98: Kulikova et. al., 2005) and Korean duck populations (0.91: Jin et. al., 2014) while the average nucleotide diversity observed in this study was found to be 0.315, which is higher than what was reported by Kulikova et. al. (2005) and Jin et. al. (2014) value of 0.83 and 0.01 respectively. In general, the nucleotide diversity of Nigerian ducks population was high, and average nucleotide difference (K) was greater compared to nucleotide diversity and this indicated a high genetic diversity as well as suggested that the populations hasn't undergone neither selection nor population expansion i.e. they still conserve their genetic uniqueness.

The observed positive Tajima's *D* indicated that the Nigerian duck populations has never departed from equilibrium, and may be as a result of lack of past/recent population expansion, bottleneck effect or heterogeneity of mutation rates (Tajima, 1996). However, the observed positive Fu's *F* value provides a strong evidence of population stationary, which may be due to absence of genetic hitchhiking, background selection and evolutionary force to drive a population expansion signature (Okello et. al., 2005; Joshi et. al., 2013).

The mtDNA phylogeny and network analysis of Nigerian duck populations revealed two divergent haplotype groups, group A and group B. Mallard ducks *(Anas platyrhynchos*) attributed to the group A haplotype clade are the most common duck species while spot-billed ducks (*Anas zonorhyncha*) formed a separate subclade nested within group A clade. The group B haplotype clade occur at high frequencies throughout Nigeria, and more related to the Muscovy duck (*Cairina moschata*) species. This suggested that Nigeria duck populations originated from two maternal lineage; the hybrid from *Anas platyrhynchos* and *Anas zonorhyncha*, and the Muscovy duck respectively. The relatively high bootstrap values of NJ tree indicated a clear genetic sub-structuring in Nigeria duck populations which could be attributed to relatively low non-random genetic intermixing between the duck populations.

The hierarchical analysis of molecular variance (AMOVA) indicated that 36.68% of maternal genetic differentiation in Nigerian duck populations resulted from variation among populations while 63.32% was due to contribution by genetic divergence among individual within population. This observed result could be as a result of hybridization which occurred among closely related species (Tubaro and Lijtmaer, 2002). Hybridization and introgression in vertebrates including birds are followed by reticulate evolution, which can substantially explain the adaptation of the allele frequencies in species and individuals (Kulikova et. al., 2004).

Conclusion

Based on the study results, Nigerian duck populations are well differentiated into two maternal lineage: the Muscovy, and the hybrid of Mallard and Spot-billed duck species. To maintain this diverse valuable duck breed, molecular markers for breed discrimination would be very beneficial for the establishment of conservation breeding program as well as aid marker assisted selection.

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