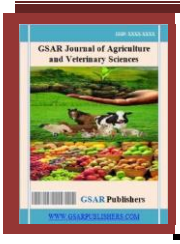

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Mitochondrial D-Loop Diversity and Morphometric Differentiation Among Plumage Phenotypes of Nigerian Local Chickens

By

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Abstract

Nigerian indigenous chicken populations carry adaptive genetic variation accumulated over centuries of natural and farmer-driven selection, and that variation is most visible in five widely recognized plumage types: Naked Neck, Frizzle feather, Normal feather, Feathered shank, and Crested. One hundred birds of five different feather types were sampled in Kwara State, Nigeria and ten morphometric linear body measurements was recorded for each bird. Blood was drawn for DNA isolation, after which the displacement loop was amplified with chicken-specific primers and sequenced in a subset of 15 birds, three per phenotypes. The results shows that Males and females differed significantly in body height ($p = 0.007$), body length ($p = 0.001$), drumstick length ($p = 0.005$), and shank diameter ($p = 0.002$). Naked Neck birds recorded the largest body dimensions on most traits and Frizzle birds the smallest. Feather type separated the groups most sharply on keel length, where Naked Neck birds (18.99 cm) exceeded Crested birds (9.35 cm) by 103.1 percent. The 15 sequences resolved six haplotypes across four polymorphic sites, giving moderate haplotype diversity ($Hd = 0.819 \pm 0.064$) on a low nucleotide diversity of $\pi = 0.00213$ per site. Neutrality statistics gave no signal of recent expansion or selection. All five phenotypic groups warrant conservation, and the nuclear architecture underlying them warrants mapping.

Keywords: *mitochondrial D-loop; Nigerian indigenous chickens; genetic diversity; plumage phenotypes; haplotype diversity; haplogroup D; conservation genetics*

1. INTRODUCTION

Village poultry underpin rural economies throughout Africa, supplying affordable animal protein and cash income while demanding little in the way of inputs (Adebambo, 2002). Indigenous birds account for a large share of the Nigerian national flock, with estimates of around 80 percent of the total poultry population (Adebambo, 2002; Oleforuh-Okoleh *et al.*, 2014). Their phenotypic range is unusually wide, most conspicuously in plumage and general morphology (Adebambo *et al.*, 2009). Centuries of natural and farmer-imposed selection have left them suited to local conditions, with useful levels of disease resistance and tolerance of the heat and humidity typical of the tropics (Odubote, 1994).

Five phenotypic groups are recognized in the Nigerian population, each named for its plumage: Naked Neck, Frizzle feather, Normal feathered, Feathered shank, and Crested (Nwosu et al., 1985; Odubote, 1994). Each is governed by a major gene, and several of those genes appear to pay their way in the tropics, where birds contend with high temperatures, swings in humidity, and a heavy disease load (Fayeye et al., 2006). These populations are the product of many generations of selection, some of it natural and some of it exercised by farmers with their own priorities.

The populations are also being eroded, and the mechanisms are well documented. Exotic commercial lines are crossed into village flocks or replace them outright, and breeding proceeds without any stated selection objective, so variation is lost without any deliberate decision (Hoffmann, 2010; Oleforuh-Okoleh et al., 2014). Naked Neck and Frizzle birds face a second and less familiar pressure. Their unusual appearance makes them preferred for sacrificial and ritual use, so they are culled disproportionately, and the phenotypes carrying the most distinctive biology are the ones disappearing fastest (Odubote, 1994).

The displacement loop of the mitochondrial genome has proved informative for questions of diversity, evolutionary

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relationship, and maternal ancestry in livestock, chickens included (Brown et al., 1982; Miao et al., 2013). Because this segment accumulates substitutions at roughly five to ten times the rate of nuclear DNA, it resolves comparatively recent events and fine-scale population structure that nuclear markers may miss (Brown et al., 1982; Clayton, 2000). Transmission is maternal and free of recombination, so lineages can be traced directly and evolutionary histories reconstructed with little ambiguity (Avise, 2000). That same property is the weakness of the marker: the D-loop resolves only the maternal half of the pedigree and is silent on the paternal contribution, a limitation that carries real weight for the question addressed here. Chicken populations across Africa, Nigerian birds among them, fall largely within haplogroup D, which places their maternal ancestry in the Indian subcontinent and implies arrival in Africa along historical trade routes (Muchadeyi *et al*., 2008; Al-Jumaili *et al*., 2020).

Earlier mtDNA surveys of Nigerian village chickens (Adebambo *et al*., 2009; Adebambo *et al*., 2010; Lasagna *et al*., 2020; Al-Jumaili *et al*., 2020) leave important questions open, in particular how maternal genetic variation relates to plumage phenotype and to the major genes that produce those phenotypes. Resolving the genetic composition of each phenotypic group matters for conservation strategy and for breeding schemes intended to raise productivity without eroding the adaptive variation these birds carry (Groeneveld *et al*., 2010; Hoffmann, 2010).

The question addressed here is whether the five plumage types of the Nigerian local chicken represent distinct maternal lineages, or whether the externally visible variation is written in the nuclear genome alone. To approach it, live weight and ten linear morphometric traits were measured across the phenotypes, the mitochondrial D-loop was sequenced to estimate diversity and score the segregating single nucleotide polymorphisms (SNPs), and the resulting haplotypes were placed on a phylogeny alongside chicken sequences of varied geographic origin.

2. Materials and Methods

2.1. Study area and sampling

Fieldwork took place in Ilorin, Kwara State, Nigeria (8°30′ N, 4°33′ E), within the North-Central geopolitical zone. The area sits in the ecological transition between Guinea and Sudan savanna. Rainfall is markedly seasonal, wet from April to October and dry from November to March, averaging 1,200 to 1,500 mm annually, while temperatures range from 23 to 37 °C (Ajadi et al., 2011). This transitional setting permits gene flow between the northern Fulani and southern Yoruba chicken ecotypes, so birds sampled here are expected to represent the principal Nigerian indigenous lineages.

Sampling was stratified, with random selection within strata. Three villages inside a 30 km radius of Ilorin were selected because they still held indigenous flocks, could be reached for repeat visits, and had taken in little exotic germplasm. Five to seven households were then drawn at random from each village register. Collection was capped at two birds per household in order to limit the inclusion of close relatives, and selection was spread across households, age cohorts, and family lines for the same reason. Birds were assigned to phenotypes in the field against standardized morphological criteria.

2.2. Study population

One hundred Nigerian local chickens (48 males, 52 females) were sampled across five phenotypic categories: (1) Normal feathered (wild type), serving as the phenotypic baseline; (2) Naked Neck (Na/Na or Na/na+), showing reduced feathering on the neck and variably on the breast; (3) Frizzle feathered (F/f+), showing the characteristic curled feathers; (4) Feathered shank (ptilopody; Pti/pti+), showing feathers on the shanks and feet; and (5) Crested (Cr/cr+), showing elongated crown feathers that form a distinct head crest. Genotypes were inferred from phenotype rather than determined molecularly, so the assignments above should be read as the most probable genotype at each locus.

The five groups were sampled at approximately equal size: Naked Neck (n = 21), Frizzle (n = 20), Feathered shank (n = 20), Crested (n = 20), and Normal feathered (n = 19). Because Naked Neck, Frizzle, Feathered shank, and Crested birds occur at frequencies of roughly 1 to 5 percent in Nigerian village flocks while Normal feathered birds account for 75 to 85 percent (Fayeye *et al*., 2006), this design deliberately over-samples the rare phenotypes. Group sizes here therefore carry no information about phenotype frequencies in the source population, and the sample should not be read as a random draw from it.

2.3. Morphometric data collection

Phenotypic characterization drew on ten linear body measurements together with live weight, taken according to established protocols (Sola-Ojo *et al*., 2009). Live weight was recorded in the morning before feeding on a calibrated top-loading scale (Mettler Toledo, USA) to the nearest 0.01 kg. Linear traits were taken with a tailor's measuring tape to the nearest 0.1 cm, and shank diameter with a digital caliper to the nearest 0.1 mm. Traits recorded comprised body weight (BDW), body height (BDH), body length (BDL), body girth (BDG), shank length (SHL), drumstick length (DSL), thigh length (THL), back-to-keel length (BKL), keel length (KEL), wing length (WGL), and shank diameter (SKD).

2.4. Blood sample collection and DNA extraction

Birds were restrained with a modified toweling technique that left only the wing exposed, which keeps handling time short and the bird calm. Blood was collected from the brachial vein in volumes of 100 to 150 µL into EDTA tubes and transported to the Molecular Unit, Department of Animal Production, University of Ilorin, for processing. Genomic DNA was extracted from whole blood using the Zymo Quick-DNA Miniprep Plus Kit (catalog number D3024, Zymo Research, USA) following the instructions of the manufacturer. DNA quantity and purity were assessed on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and samples yielding 30 to 50 ng/µL were taken forward

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to amplification at the University College Hospital, Ibadan, Nigeria.

2.5. PCR amplification and sequencing

Amplification targeted the displacement loop of the chicken mitochondrial genome using chicken-specific primers (Liu *et al*., 2006), forward 5′-TTGGCCTTTCTCCAAGGACT-3′ and reverse 5′-AGCAAGTCGTGCTAGGGTGT-3′. Each 25 µL reaction combined 50 to 100 ng of template DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, and 1.0 unit of Taq DNA polymerase (Miao et al., 2013). Cycling opened with a 3 min denaturation at 94 °C, ran through 35 cycles of 94 °C for 30 s, 55 to 60 °C for 30 s, and 72 °C for 1 min, and closed with a 7 min extension at 72 °C. Amplicons were checked on 1.5 percent agarose gels. Products that amplified cleanly were purified and read by Sanger sequencing on an ABI 3500 Genetic Analyzer (Applied Biosystems, USA), with both strands sequenced so that base calls could be confirmed, three birds per feather phenotype were drawn at random within each group for sequencing.

2.6. Statistical analysis for Morphometric Body Parameters

Morphometric records were analyzed by two-way analysis of variance (ANOVA), with feather type and sex fitted as fixed effects and their interaction included in the model. Means were separated by Duncan's multiple range test wherever the ANOVA returned a significant effect. Correlations among morphometric traits were examined within each feather type group. Results are reported as means with their standard deviations, and effects were treated as significant at $p < 0.05$. No adjustment was made for multiple testing across the eleven traits, so p-values close to the threshold should be interpreted with that in mind. SPSS version 20 (IBM Corp., Armonk, NY) was used for all phenotypic analyses.

2.7. Mt D-Loop Sequence analysis and genetic diversity

Chromatograms were inspected and aligned in BioEdit (Hall, 1999) using ClustalW (Thompson *et al*., 1994) in order to score polymorphic sites and assign haplotypes. Diversity indices were computed in DnaSP version 6.12.03 (Rozas et al., 2017). Haplotype diversity (Hd) expresses the probability that two haplotypes drawn at random from the sample differ (Nei, 1987), while nucleotide diversity (π) gives the mean per-site difference between pairs of sequences (Nei and Li, 1979). Departures from mutation-drift equilibrium were tested with Tajima's D, Fu and Li's D* and F*, and Fu's Fs (Tajima, 1989; Fu, 1997). Trees were built in MEGA version 11 under neighbor-joining and maximum-likelihood criteria (Tamura *et al*., 2021).

2.8. Ethics Statement

The work followed the ARRIVE guidelines for reporting animal research (Percie du Sert et al., 2020). Sampling was carried out in the cooler parts of the day to limit heat stress. Birds were restrained for no longer than 3 minutes using a modified toweling technique, and blood was drawn from the brachial vein with a 25-gauge needle after cleaning the site with 70 percent ethanol. The volumes taken, 100 to 150 µL, represent less than 0.1 percent of total blood volume and fall well below accepted limits for birds of this size. Each bird was observed for at least 5 minutes after collection, and no adverse events were recorded. The institutional review body of Kwara State University, Malete, approved the study before sampling began, and every participating farmer gave verbal informed consent before their birds were sampled.

3. Results

3.1. Sexual dimorphism in morphometric traits

Across the 100 birds measured (48 males, 52 females), traits varied widely (Table 1). Males were the larger sex on most skeletal measurements. The clearest separations were in body length (37.09 cm in males against 33.70 cm in females, $p = 0.001$) and body height (30.33 cm against 27.72 cm, $p = 0.007$). Drumstick length ran 12.47 cm in males and 11.21 cm in females, a gap of 11.2 percent ($p = 0.005$). Shank diameter gave the widest proportional difference, 40.1 mm against 35.5 mm, so males carried shanks about 13.0 percent thicker ($p = 0.002$). Body weight showed a weaker pattern, with males averaging 1.158 kg and females 1.059 kg, a difference that did not reach significance ($p = 0.156$). Body girth ($p = 0.055$) and shank length ($p = 0.075$) fell just outside the significance threshold and are reported as non-significant.

Table 1.0. Sexual dimorphism in morphometric traits of Nigerian local chickens (n = 100).

Morphometric trait	Males (n = 48) Mean ± SD	Females (n = 52) Mean ± SD	Mean difference	p-value	Significance
Body weight (kg)	1.158 ± 0.339	1.059 ± 0.357	0.099	0.156	NS
Body height (cm)	30.33 ± 4.61	27.72 ± 4.79	2.61	0.007	**
Body length (cm)	37.09 ± 5.39	33.70 ± 4.81	3.39	0.001	**
Body girth (cm)	30.02 ± 4.56	28.32 ± 4.18	1.70	0.055	NS
Shank length (cm)	8.49 ± 1.86	7.80 ± 1.93	0.69	0.075	NS
Drumstick length (cm)	12.47 ± 2.34	11.21 ± 2.10	1.26	0.005	**

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Morphometric trait	Males (n = 48) Mean ± SD	Females (n = 52) Mean ± SD	Mean difference	p-value	Significance
Thigh length (cm)	10.19 ± 1.69	9.79 ± 1.97	0.40	0.277	NS
Keel length (cm)	13.06 ± 5.79	12.20 ± 5.12	0.86	0.432	NS
Wing length (cm)	14.10 ± 3.25	13.30 ± 3.68	0.80	0.251	NS
Back-to-keel length (cm)	2.51 ± 0.58	2.37 ± 0.44	0.14	0.162	NS
Shank diameter (mm)	40.1 ± 7.8	35.5 ± 6.4	4.6	0.002	**

*** p < 0.01; NS, not significant at p < 0.05. Body girth (p = 0.055) and shank length (p = 0.075) approached the threshold without reaching it. Mean difference is males minus females. Shank diameter is reported in millimeters, consistent with caliper measurement.*

3.2. Phenotypic variation by feather type

Grouping the same measurements by feather type produced sharper contrasts than sex did (Tables 2 and 3). For most traits the spread among feather types was the larger source of variation, which suggests that the major plumage genes act well beyond the feather coat and shape body conformation itself.

Naked Neck birds were at the top of the range for body weight, body length, keel length, and shank diameter (Table 3), while Frizzle birds anchored the bottom of the range on most traits, including the shortest body height at 25.90 cm. Body height was the exception to the Naked Neck pattern, with Feathered shank birds the tallest at 31.99 cm. Keel length was the trait on which the phenotypes separated most: 18.99 cm in Naked Neck birds against 9.35 cm in Crested birds, a difference of 103.1 percent (p = 0.003). A separation of that magnitude in a single skeletal trait would, if confirmed, point to pleiotropic effects of the Na gene on skeletal development extending beyond its recognized thermoregulatory role (Horst, 1988; Fathi et al., 2013). Body weight differed by 47.7 percent between Naked Neck (1.329 kg) and Frizzle (0.900 kg) birds, well above the 9.3 percent difference attributable to sex in the same trait.

Table 2.0. Effects of feather type on morphometric traits (two-way ANOVA).

Trait	F-value	p-value	Most different groups	Difference between extreme groups
Body weight	3.91	0.008	Naked Neck > Frizzle	47.7%
Body height	3.42	0.019	Feathered shank > Frizzle	23.6%
Body length	2.54	0.059	Naked Neck > Feathered shank	12.1%
Body girth	2.18	0.082	Normal > Frizzle	19.4%
Shank length	2.11	0.097	Feathered shank > Frizzle	38.1%
Drumstick length	2.42	0.067	Naked Neck > Crested	24.3%
Thigh length	2.06	0.104	Normal > Frizzle	not reported
Keel length	5.17	0.003	Naked Neck > Crested	103.1%
Wing length	3.21	0.020	Normal > Frizzle	44.1%
Shank diameter	2.89	0.032	Naked Neck > Frizzle	23.0%

Effects were treated as significant at p < 0.05. Body length, body girth, shank length, drumstick length, and thigh length did not reach the threshold and are retained so that the full set of traits tested is visible. The final column gives the percentage difference between the two extreme group means and is descriptive rather than a formal effect size. Back-to-keel length was measured but not tested by feather type.

Table 3. Morphometric means by feather type, with Duncan groupings.

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Feather type (n)	Body weight (kg)	Body height (cm)	Body length (cm)	Keel length (cm)	Wing length (cm)	Shank diameter (mm)
Naked Neck (21)	1.329 ± 0.28 c	29.88 ± 2.76 b	37.62 ± 4.12 b	18.99 ± 5.78 c	13.87 ± 2.89 b	42.4 ± 8.2 c
Normal (19)	0.992 ± 0.22 ab	29.05 ± 3.42 b	37.00 ± 3.81 b	10.43 ± 1.88 ab	16.56 ± 4.02 c	38.3 ± 7.4 b
Frizzle (20)	0.900 ± 0.18 a	25.90 ± 2.13 a	34.45 ± 2.98 ab	12.93 ± 3.21 b	11.50 ± 1.54 a	34.5 ± 6.2 a
Feathered shank (20)	1.215 ± 0.31 c	31.99 ± 3.15 c	33.59 ± 2.11 a	10.93 ± 2.15 ab	14.78 ± 3.01 b	36.1 ± 7.1 ab
Crested (20)	1.080 ± 0.24 b	28.00 ± 2.89 ab	33.95 ± 2.67 ab	9.35 ± 1.02 a	11.85 ± 2.12 a	37.2 ± 5.8 ab

Means within a column that share a letter do not differ at $p < 0.05$ by Duncan's multiple range test. Body girth, shank length, drumstick length, thigh length, and back-to-keel length were measured, but group means for those traits are not yet available.

Table 4. Sexual dimorphism compared with feather type effect, by trait.

Trait	Sexual dimorphism (%)	Feather type effect (%)	Larger source of variation
Body weight	9.3	47.7 (Naked Neck vs Frizzle)	Feather type
Body height	9.4	23.6 (Feathered shank vs Frizzle)	Feather type
Body length	10.1	12.1 (Naked Neck vs Feathered shank)	Comparable
Body girth	6.0	19.4 (Normal vs Frizzle)	Feather type
Keel length	7.0	103.1 (Naked Neck vs Crested)	Feather type
Drumstick length	11.2	24.3 (Naked Neck vs Crested)	Feather type
Shank length	8.8	38.1 (Feathered shank vs Frizzle)	Feather type
Wing length	6.0	44.1 (Normal vs Frizzle)	Feather type
Shank diameter	13.0	23.0 (Naked Neck vs Frizzle)	Feather type

Percentages express the difference between the two extreme group means relative to the smaller mean. Thigh length and back-to-keel length are omitted because feather type group means were not available for them.

3.3. Mitochondrial D-loop genetic diversity

The 15 sequenced birds, drawn from all five phenotypic groups, carried moderate haplotype diversity on very little nucleotide variation (Table 5.0). Of the 589 aligned positions, 13 carried alignment gaps or missing data and were excluded, leaving 576 sites for analysis. Of these, 572 were invariable (99.3 percent) and 4 were polymorphic, carrying 5 mutations in total: two singleton sites and two parsimony-informative sites. GC content was 49.5 percent, consistent with published chicken mtDNA composition (Desjardins and Morais, 1990).

Six haplotypes were resolved from the 15 individuals (Hd = 0.819 ± 0.064). Nucleotide diversity was $\pi = 0.00213$ per site, with an average of 1.229 pairwise differences. The four polymorphic sites lie at positions 8, 10, 12, and 331 of the amplicon (Figure 1). Position 8 carried two variants and was parsimony-informative, position 331 likewise; position 10 carried a single variant in one bird; and position 12 carried three variants, accounting for the fifth mutation. Everything between positions 13 and 330, and between 332 and 577, was identical in every bird, which follows arithmetically from the position of the four variable sites rather than constituting an independent observation.

The distribution of those sites is the most consequential feature of the sequence data. Three of the four polymorphic positions, and four of the five mutations, fall within the first 12 bases of the read. Removing positions 8, 10, and 12 and retaining only position 331 reduces the dataset from six haplotypes to two and lowers haplotype diversity from 0.819 to 0.419. The reported haplotype structure therefore depends almost entirely on three bases situated where Sanger base calling is least reliable.

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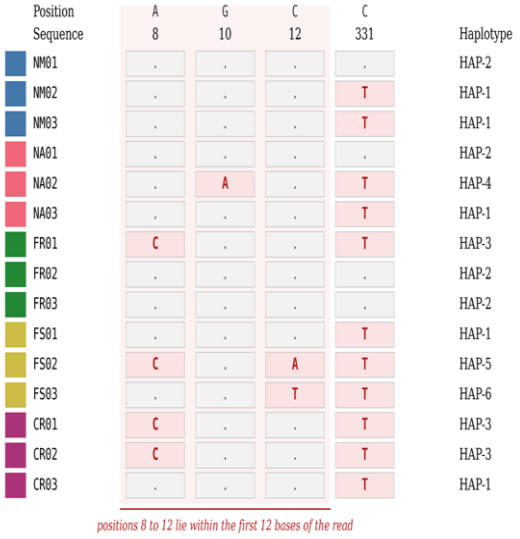

Figure 1. Polymorphic sites in the mitochondrial D-loop of 15 Nigerian local chickens. Columns give the four variable positions and rows the individual birds, grouped and color-coded by plumage phenotype. Dots indicate identity with the reference sequence NM01 and letters indicate substitutions. Resulting haplotype assignments are given at right. Positions 8, 10, and 12 lie within the first 12 bases of the amplicon.

Table 5. Mitochondrial D-loop sequence analysis summary statistics.

Parameter	Value	Interpretation
Sequences analyzed	15	Drawn from 5 phenotypes
Aligned length	589 bp	D-loop region
Sites with gaps or missing data	13	Excluded from analysis
Sites analyzed	576	Basis for all polymorphism counts
Polymorphic sites (S)	4	Low; indicates high conservation
Total mutations (Eta)	5	One site carries two mutations
Singleton variable sites	2	Restricted to one sequence each
Parsimony-informative sites	2	Available for phylogenetic inference
Number of haplotypes (h)	6	Moderate for 15 sequences
Haplotype diversity (Hd)	0.819 ± 0.064	Moderate (range 0 to 1)
Nucleotide diversity (π)	0.00213 per site	Low; typical for village poultry
Theta from segregating sites	0.00214 to 0.00268	Close to π, consistent with equilibrium
Average pairwise differences (k)	1.229	Low sequence divergence
Invariable sites	572 of 576 (99.3%)	Low variation overall
Polymorphic site positions	8, 10, 12, 331	Three sites within the first 12 bases
GC content	49.5%	Normal for chicken mtDNA
Recombination events (Rm)	0	Expected for maternally inherited mtDNA

3.4. Haplotype distribution by feather phenotype

No haplotype was restricted to a single feather phenotype among those occurring more than once (Table 6). The most common haplotype, HAP-1 (33.3 percent), was carried by Normal, Naked Neck, Feathered shank, and Crested birds; HAP-2 (26.7 percent) by Normal, Naked Neck, and Frizzle birds; and HAP-3 (20.0 percent) by Frizzle and Crested birds. The three remaining haplotypes were each represented by a single bird, HAP-4 in a Naked Neck bird and HAP-5 and HAP-6 in Feathered shank birds, and a singleton cannot

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demonstrate phenotype specificity. The shared distribution is consistent with recent divergence of the phenotypes from a narrow set of maternal lineages, although three sequences per group give limited power to detect phenotype-specific haplotypes even if they exist.

Table 6. Haplotype distribution by feather phenotype.

Haplotype	Bases at positions 8, 10, 12, 331	n	Phenotypic distribution	Frequency
HAP-1	A G C T	5	Normal (2), Naked Neck (1), Feathered shank (1), Crested (1)	33.3%
HAP-2	A G C C	4	Normal (1), Naked Neck (1), Frizzle (2)	26.7%
HAP-3	C G C T	3	Frizzle (1), Crested (2)	20.0%
HAP-4	A A C T	1	Naked Neck (1)	6.7%
HAP-5	C G A T	1	Feathered shank (1)	6.7%
HAP-6	A G T T	1	Feathered shank (1)	6.7%

Haplotypes are defined entirely by the four polymorphic positions. Positions 8, 10, and 12 fall within the first 12 bases of the read; see Section 4.2.

3.5. Neutrality tests and population structure

None of the neutrality statistics departed significantly from neutral expectations (Table 7). Tajima's D was -0.678 ($p > 0.10$). Fu and Li's D* (-1.004) and F* (-1.049) likewise fell short of significance, as did Fu's Fs at -2.184 ($p > 0.10$). Linkage disequilibrium was minimal. Across the three pairwise comparisons available among the three biallelic sites, ZnS was 0.061 and Za 0.026, with Wall's B and Wall's Q both zero and no recombination detected (Rm = 0), as expected for a uniparentally inherited genome (Avise, 2000).

Table 7. Neutrality test statistics for Nigerian local chicken mtDNA.

Neutrality test	Statistic value	p-value	Interpretation
Tajima's D	-0.678	$p > 0.10$	No departure from equilibrium detected
Fu and Li's D*	-1.004	$p > 0.10$	No departure from equilibrium detected
Fu and Li's F*	-1.049	$p > 0.10$	No departure from equilibrium detected
Fu's Fs	-2.184	$p > 0.10$	No signal of recent demographic expansion
Achaz's Y	0.296	not significant	Consistent with neutrality
Strobeck's S	0.971	$P(k \leq 6) = 0.072$	Haplotype number close to neutral expectation

All tests have low power at n = 15, so these results indicate an absence of evidence rather than evidence of absence. Values for Fu and Li's D and F* are those reported by DnaSP for the full dataset. An earlier draft quoted -0.01225 and 0.03353, which are the statistics from the sub-analysis restricted to the three biallelic positions, alongside Achaz's Y*.*

3.6. Phylogenetic analysis and haplogroup classification

The median-joining network resolves to a single minimum spanning topology, since with four variable sites no alternative connection of equal length exists and no median vectors are required (Figure 2). The network is star-like, centered on HAP-1, the most frequent haplotype and the only one carried by four of the five phenotypes. HAP-2, HAP-3, HAP-4, and HAP-6 each attach to HAP-1 by a single mutational step, and HAP-5 attaches to HAP-3, so the greatest separation between any two haplotypes is three steps. That topology is consistent with recent common ancestry and limited subsequent divergence, which matches the low nucleotide diversity recorded here.

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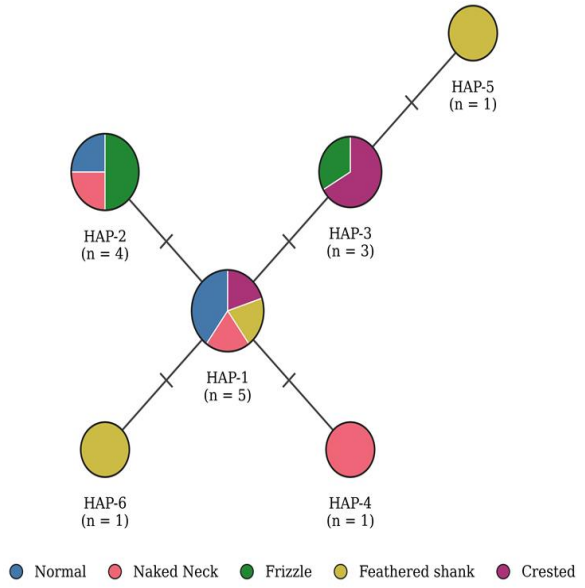

Figure 2. Median-joining network of the six mitochondrial D-loop haplotypes recovered from Nigerian local chickens. Circle area is proportional to haplotype frequency and sectors give the plumage phenotypes contributing to each haplotype. Hatch marks on connecting branches denote single mutational steps. With four variable sites the minimum spanning topology is unique and no median vectors are required.

4.0. Discussion

4.1. Sexual dimorphism and phenotypic variation

That males were larger on most traits is expected and matches earlier work on Nigerian village chickens (Fayeye *et al.*, 2006, Adebambo *et al.*, 2009 and Sola-Ojo *et al*., 2010). The shank diameter difference of 13.0 percent probably reflects sex differences in bone robustness and dermal thickening, with cocks developing heavier shank architecture to carry their spurs and greater mass. For 8 of the 9 traits where the two could be compared directly, the difference between the extreme feather type groups exceeded the difference between the sexes. Shank diameter was the exception, where the sexual dimorphism of 13.0 percent was comparable to the feather type effect of 23.0 percent. This pattern is consistent with the major plumage genes exerting pleiotropic effects on overall body conformation.

Naked Neck birds ranked highest on several traits, which fits the view that the dominant Na gene raises overall growth and body size as reported by Peters *et al.*, 2011; Fathi *et al*., 2013). This is in contrary to Fayeye *et al*. (2006), who found Naked Neck birds no better than Normal-feathered birds on most measurements, this could be attributed to sample population and other environmental factor. Frizzle birds showed the smallest measurements for body height and several other traits, which is consistent with metabolic costs associated with the effects of the F gene on feather structure and thermoregulation as reported by Sharifi *et al*., 2010. The frizzle condition shows incomplete dominance while heterozygotes display moderate curling with good viability, homozygotes show extreme curling associated with reduced fertility and survival (Horst, 1988; Sharifi et al., 2010). That contrast in fitness between the two genotypes offers a mechanism by which the polymorphism persists in village flocks despite the reduced average growth of frizzled birds.

4.2. Mitochondrial genetic diversity and population structure

Moderate haplotype diversity (Hd = 0.819) combined with very low nucleotide diversity (π = 0.00213 per site) is a pattern characteristic of chicken populations that passed through founder events during domestication and subsequent dispersal (Liu et al., 2006; Miao et al., 2013). The value recorded here is lower than reported for Indian populations (π = 0.008 to 0.012) and Southeast Asian populations (π = 0.006 to 0.010), which matches the reduced diversity generally described for African indigenous chickens (Liu et al., 2006; Muchadeyi et al., 2008). It is also lower than the 0.02 reported for 171 Nigerian birds over a 397 bp fragment (Adebambo *et al.*, 2017), a difference large enough that the two datasets should be compared over a common region before the contrast is interpreted. Two mechanisms are usually invoked: a small number of founder birds reaching the continent along Asian trade routes, and relative isolation of the populations that grew from them. Zimbabwean (Muchadeyi *et al*., 2008) and Ugandan (Yussif *et al.*, 2024) flocks show comparably low values, so the pattern in Kwara appears to reflect the general phylogeographic signature of sub-Saharan African village chickens rather than a local peculiarity.

The position of the four polymorphic sites requires comment before any of this is interpreted. Three of them, at positions 8, 10, and 12, sit within the first 12 bases of the amplicon, and they carry four of the five mutations. Sanger sequencing produces its least reliable base calls at the start of a read, where the trace is compressed and the fluorescence signal has not stabilized, and it is standard practice to trim that segment before analysis. The haplotype structure reported here shows that maternal diversity is moderate rather than very low. The two long invariant stretches at positions 13 to 330 and 332 to 577 carry no independent information. They are simply the intervals between the four variable positions, and they would appear in any dataset with this few polymorphic sites regardless of the underlying biology.

Earlier work places the maternal ancestry of Nigerian village chickens predominantly in haplogroup D, which originates in the Indian subcontinent (Adebambo et al., 2010; Adebambo et al., 2017), although Lasagna et al. (2020) assigned all 96 Yoruba and Fulani sequences they examined to haplogroup E, so the picture for Nigeria is not uniform. Assigning the present haplotypes to a haplogroup requires alignment against reference sequences of known haplogroup membership. Thus, sampling of a single state with 15 sequences would in any case be unable to distinguish a single introduction from several closely related ones. Tajima's D of -0.678 leaves the maternal lineages close to equilibrium expectations. Fu's Fs, were also non-significant at -2.184, points the same way, with haplotype frequencies resembling those of a population

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broadly stable since founding, an event that trade route evidence places roughly 2,000 to 3,000 years ago as stated by Muchadeyi *et al*. (2008) and Mwacharo *et al*. (2011).

4.4. Phenotype-genotype discordance and conservation implications

Reading the two datasets together shows a mismatch. The nuclear side is active, producing five distinct phenotypes under the control of major genes, while the maternal side is comparatively quiet, with mtDNA diversity at $\pi = 0.00213$. All five phenotypes fall inside haplogroup D, and no haplotype was confined to one phenotype. The most economical reading is that the phenotypic range was built by mutation and selection acting on nuclear variation, on top of a founder population carrying a narrow set of maternal lineages. A small maternal base supporting a wide nuclear range is what published domestication models for African chickens would predict (Liu *et al.*, 2006; Miao *et al*., 2013). Sampling maternal lineages would inflate the apparent coverage while protecting very little, since all five phenotypes trace to the same narrow maternal base. Each phenotypic group therefore has to be conserved in its own right, including the Naked Neck and Frizzle birds that are culled for sacrificial and cultural reasons, because that is where the adaptive variation is held. Breeders can meanwhile make use of the body size differences, the Naked Neck advantage in particular, provided flock-level diversity is monitored rather than size alone as suggested by Groeneveld *et al.*, (2010) and Hoffmann, (2010).

5.0. Conclusion.

This results shows that the plumage genes are associated with far more than plumage, with keel length differing by 103.1 percent between Naked Neck and Crested birds and body weight by 47.7 percent between Naked Neck and Frizzle birds and differences was beyond sex effects. Six haplotypes among 15 birds gave Hd = 0.819, nucleotide diversity sat at $\pi = 0.00213$ per site, every sequence fell within haplogroup D, and no haplotype was confined to a single phenotype. The morphometric conclusions, resting on 100 birds, stand on firmer ground, although they too would benefit from a repeat measurement protocol given the variability recorded for keel length. This work shows that conserving maternal lineages will not conserve what makes these birds worth keeping. The useful variation sits in the nuclear genome, spread across all five phenotypic groups, and mapping it properly will require microsatellite or SNP-array work. That is the study needed next, and keel length is where it should start.

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