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Genetic Characterization of the Cuban Creole Gamecock Using the Mitochondrial DNA D-loop

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ABSTRACT

Background: One of the greatest global challenges is the extinction of species and breeds for various reasons, including the loss of genetic resources. These phenomena result from the depletion of genetic resources caused by intensive production systems, genetic selection, hostile climates, and the introduction of non-native species into new habitats. **Aim** To genetically characterize the Cuban Creole gamecock (*Gallus gallus*) using the mitochondrial DNA D-loop region. **Materials and Methods:** The study was conducted in private breeding farms belonging to breeders associated with the National Wildlife Protection Company in the western, central, and eastern regions of Cuba. A total of 53 birds were randomly selected and feather samples were collected for DNA extraction, purification, and amplification. Sequencing was performed by Macrogen Inc. The obtained sequences comprised 578 base pairs. Sequence alignments were performed with ClustalX 1.83, a UPGMA tree was constructed with MEGA v.7, and diversity indices were calculated using DnaSP v.6.0. **Results:** Twenty-two haplotypes were identified. Haplotype diversity was high (0.78) and nucleotide diversity was moderate (0.00443). Tajima's D was negative and highly significant (−2.354; $P < 0.01$), indicating a departure from neutrality and suggesting an excess of low-frequency polymorphisms. **Conclusions:** The sampled birds exhibited very high genetic variability. This study represents the first mitochondrial DNA-level genetic characterization of the Cuban Creole gamecock.

Keywords: birds; haplotypes; mitochondrial DNA; sequencing (*Fuente: AIMS*)

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INTRODUCTION

One of the greatest global challenges is the extinction of species and breeds for multiple reasons. Major natural causes include loss of disease resistance and inability to adapt to certain climates. These phenomena result from the depletion of genetic resources caused by intensive production systems, genetic selection, hostile climates, and the introduction of non-native species into new habitats (Kullan *et al*., 2026). In addition, the high costs of cryopreservation of genetic material and of genetic analyses hinder the conservation of genetic resources and the conduct of related studies, as noted by the FAO (2019). Domestic birds kept for egg and meat production, for feathers, for ornamental purposes, and as fighting birds are also affected by these factors, leading to breed loss and reduced genetic diversity.

In recent years, scientists have used genetic markers to investigate the genetic structure of populations. Polymorphic mitochondrial DNA sequences in the D-loop region have been frequently employed to describe maternal lineages in animals, especially in birds (Hasan *et al*., 2016; Meydan *et al*., 2016; Kenchaiwong *et al.*, 2025). Unlike microsatellites, mitochondrial DNA is not inherited in a Mendelian manner but is transmitted exclusively through the maternal line, which greatly facilitates phylogeny reconstruction; additionally, its high mutation rate aids in identifying population origin and distribution (ADNAbuabara *et al*., 2021).

Lack of knowledge about the genetic characteristics of Cuban fighting cocks exposes them to the risk of losing valuable genes with sociocultural and economic consequences. Therefore, the purpose of this study was to genetically characterize the Cuban Creole fighting cock (*Gallus gallus*) using the mitochondrial DNA D-loop region.

MATERIALS AND METHODS

Location and duration

The study was conducted in private breeding farms of breeders affiliated with the National Wildlife Protection Company in the western, central, and eastern regions of Cuba during 2021.

Animals and farms

Feather samples with follicles were collected from 53 birds (Table 1). Animals on each farm were selected at random. Two feathers were taken from the dorsal region and two from the ventral region of each bird; samples were placed in Ziploc bags and transported in a thermos following Tauros (2019). Samples were sent to the Molecular Biology Laboratory, Faculty of Agricultural Sciences, University of Cuenca, Ecuador.

Table 1. Origin and number of birds subjected to the mtDNA study

Zone	Province	Municipality	N
West	Pinar del Río	Pinar del Río	2
		Mantua	1
		San Luis	1
	Havana	Cerro	2

		Marianao	2
		Guanabacoa	1
		Guanajay	2
	Artemisa	Güira de Melena	2
		Artemisa	1
	Mayabeque	San José	1
		Güines	2
		Santa Clara.	2
	Las Villas	Caibarien	1
		Manicaragua	1
		Santi Spiritus	2
	Santi Spiritus	Trinidad	1
		Taguasco	2
Central		Ciego de Ávila	1
	Ciego de Ávila	Morón	2
		Majagua	1
		Camagüey	2
	Camagüey	Esmeralda	1
		Guáimaro	1
		Florida	2
		Bayamo	2
	Granma	Guisa	2
		Buey Arriba	1
		Santiago de Cuba	1
	Santiago de Cuba	El Caney	1
		Palma Soriano	2
East		Holguín	2
	Holguín	Biran	2
		Sagua de Tánamo	1
		Guantánamo	1
	Guantánamo	Maisí	1
		Baracoa	1

DNA extraction and purification

Mitochondrial DNA was extracted following a standardized protocol that included follicle disruption, incubation in 70% ethanol, centrifugation, lysis with Tris-HCl, EDTA, NaCl and SDS, digestion with proteinase K and DTT, extraction with phenol/chloroform/isoamyl alcohol, precipitation with absolute ethanol, washing with 70% ethanol, and resuspension in molecular-biology-grade water. Quantification was performed using a spectrophotometer.

PCR amplification

The mitochondrial D-loop region was amplified by PCR using the oligonucleotides OligoL 16750-For and OligoCR16-Rev. The 25 µL reaction mixture contained amplification buffer 1X, enhancer solution 1X, dNTPs (0.2 mM each), $MgSO_4$ (2 mM), oligonucleotides (0.8 µM each), Pfx DNA polymerase (0.04 U/µL) and total DNA (2 ng/µL). The thermal profile included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at a gradient temperature, and extension at 68°C for 45 seconds, with a final extension at 68°C for 5 minutes (see Tables 2 and 3).

Table 2. Reagents for DNA amplification

Reagent	Concentration	Volume	Final concentration
Molecular biology grade water	N/A	12.9 µl	N/A
10X amplification buffer	10 X	2.5 µl	1X
10X enhancer solution	10 X	2.25 µl	1X
dNTP´s	10 mM ea.	0.5 µl	0.2mM ea.
MgSO4	50 mM	1 µl	2 mM
OligoL 16750-For	100 µM	0.2 µl	0.8 µM
OligoCR16-Rev	100 µM	0.2 µl	0.8 µM
Pfx DNA polymerase	2.5 U/µl	0.2 µl	0.04 U/µl
Total DNA sample	10 ng/µl	5µl	2 ng/µl
Final volume	-	25 µl	-

Table 3. Amplification thermal profile

Steps	Initial denaturing	Number of cycles 35			Final extension	Storage
		Denaturing	Alignment	Extension		
Time (min ´, sec´´)	5´	45´´	25´´	45´´	5´	
Temperature (°C)	94.0 °C	94.0 °C		68.0 °C	68.0 °C	4.0 °C

Sequencing

Sequencing was performed by Macrogen Inc. (Macrogen, 2019) using the BigDye Terminator version 3.1 kit and an ABI3730XL DNA analyzer. Fifty-three samples were submitted and all were successfully sequenced. The samples were filtered and re-sequenced four times (two replicates in the molecular biology laboratory and two by Macrogen).

Statistical analysis

The obtained sequences were 578 base pairs long. The FASTA files were edited with BioEdit (Hall, 1999) and multiple alignments were performed with ClustalX 1.83. A traditional phylogenetic tree was constructed using the UPGMA method with MEGA v.7. Genetic diversity indices included: number of segregating sites (S), number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), average number of nucleotide differences (k), and Tajima's D neutrality test. Analyses were performed using DnaSP v.6.0.

RESULTS AND DISCUSSION

Haplotypes and genetic diversity

Twenty-two haplotypes were identified among the 53 sequences analyzed (Table 4). The most frequent haplotype (Hap_2) comprised 26 individuals, while 15 individuals presented unique haplotypes. The number of polymorphic sites was 35, a value higher than reported in most of the studies consulted (Do *et al*., 2019; Yussif and Kugonza, 2023; Oni *et al*., 2025). Eltanany *et al*. (2016), Meydan *et al*. (2016), Zhuang *et al*. (2023), and Kullan *et al.* (2026) reported between 12 and 49 polymorphic sites. No deletions or insertions were found. The total number of mutations was 39, of which 27 were singletons.

Table 4. Haplotypes obtained for the Cuban Creole fighting rooster

Haplotypes	N	Animals sharing each haplotype
Hap_1	1	[1]
Hap_2	26	[2-3, 6-7, 12-14, 20, 22, 27-28, 31, 33-34, 40-41, 43-49, 51, 53]
Hap_3	1	[4]
Hap_4	1	[5]
Hap_5	2	[8, 17]
Hap_6	2	[9, 11]
Hap_7	1	[10]
Hap_8	1	[15]
Hap_9	2	[16, 21]
Hap_10	1	[18]
Hap_11	1	[19]
Hap_12	1	[23]
Hap_13	4	[24, 26, 32, 42]
Hap_14	1	[25]
Hap_15	2	[29, 50]
Hap_16	1	[30]
Hap_17	1	[35]
Hap_18	1	[36]
Hap_19	1	[37]
Hap_20	1	[38]
Hap_21	1	[39]
Hap_22	1	[52]

N: Number of samples

Haplotype diversity (Hd) was 0.78 (±0.062), a value considered high and above 0.7. This result is similar to that reported by Zhuang *et al.* (2023) in Guangxi, China (0.83) and by Kullan *et al*. (2026) in four native Chinese breeds (0.92), but higher than the values found by Meydan *et al.* (2016) in Turkey (0.33) and by Eltanany *et al.* (2016) in Egypt (between 0.56 and 0.71). Nucleotide diversity (π) was 0.00443, a low to moderate value, lower than that reported by Do *et al*. (2019) in Indonesia (0.0099) but higher than the values reported by Zhuang *et al*. (2023), Meydan *et al*. (2016), Eltanany *et al.* (2016) and Kullan *et al.* (2026), which did not exceed 0.003.

The average number of nucleotide differences (k) was 2.56, a value that is double that reported by Eltanany *et al.* (2016) (1.24) and three times that found by Meydan *et al*. (2016). Tajima's D was negative and highly significant (−2.354; $P<0.01$), indicating a deviation from neutrality, probably due to an excess of low-frequency polymorphisms and selective pressure (Table 5). This result contrasts with populations studied by Zhuang *et al*. (2023), who reported positive D values.

Table 5 Genetic diversity indices of sequences estimated for the analyzed sample of Cuban Creole fighting roosters

Breed	n	S	h	Hd	π	k	D
Cuban	53	35	22	0.78	0.00443	2.56	-2.354
± SD	-	-	-	±0.062	±0.000001	±0.941	P<0.01

n: number of sequences; S: number of polymorphic sites; h: number of haplotypes; Hd: haplotype diversity; SD: standard deviation; π: nucleotide diversity; k: average number of nucleotide differences; D: Tajima's D

Phylogenetic analysis

Figure 1 shows the tree-shaped distribution of clusters of the studied roosters is shown. Although Haplotype 2 grouped 26 individuals, Haplotype 13 grouped four; Haplotypes 5, 6, 9, and 15 each included two individuals; and 15 individuals carried unique haplotypes. The topology of the UPGMA tree suggests a complex population structure with multiple maternal lineages.

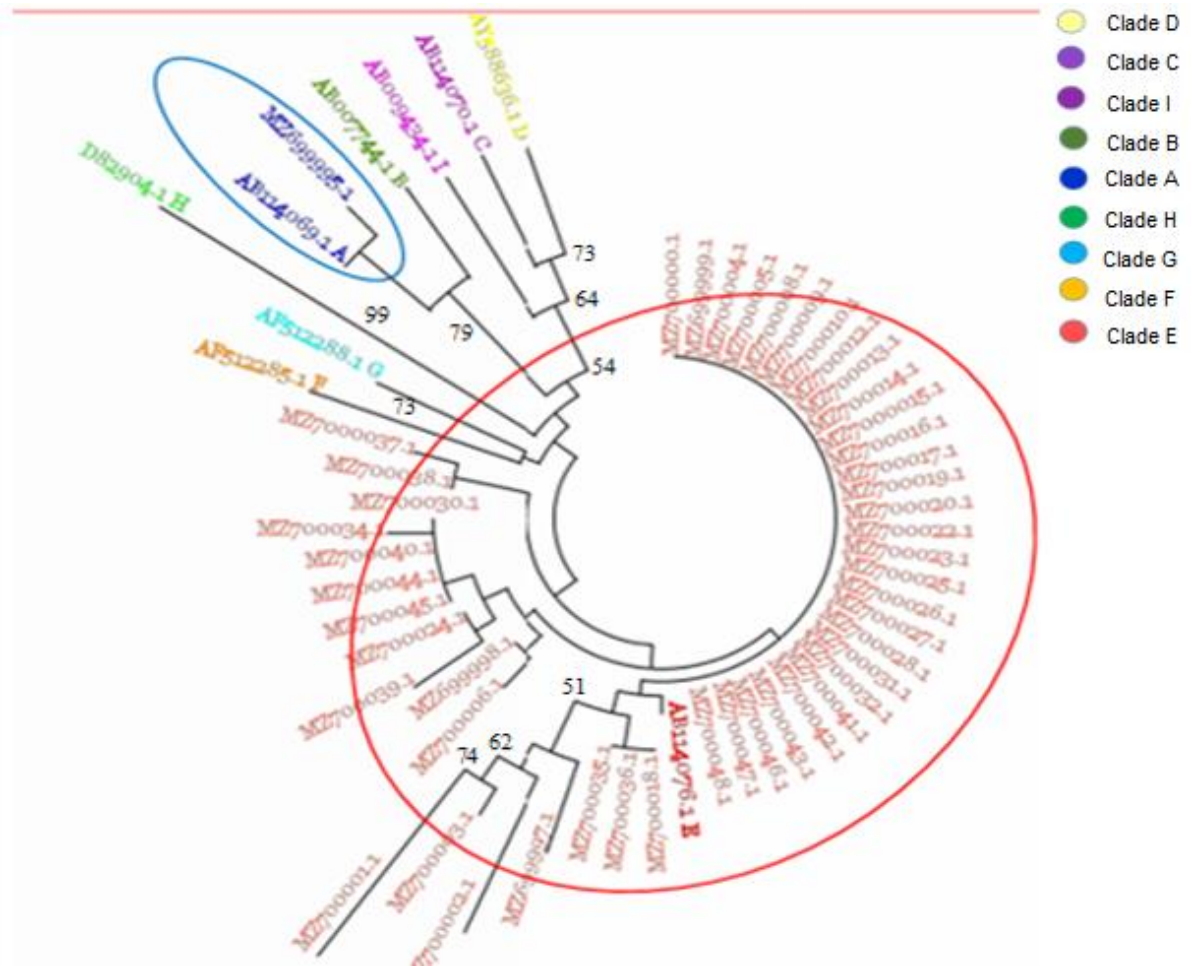

Figure 1. Neighbor-Joining phylogenetic diagram with sequence codes of Cuban roosters, arranged by the genetic clade to which they belong, rooted tree. One thousand bootstrap replicates were used. MZ699995–MZ700048 are D-loop mtDNA sequences from Cuban Creole fighting roosters. Reference sequences used were: AB114069 (clade A), AB007744 (clade B), AB003494 (clade I), AB114070 (clade C), AY588636 (clade D), AF512288 (clade G), AF512285 (clade F), D82904 (clade H). The red circle indicates samples placed in clade E and the blue circle those in clade A.

The haplotype and nucleotide diversity values found suggest a substantial contribution of the Cuban Creole fighting roosters to the species' genetic diversity, similar to that reported by Kullan *et al*. (2026) for Chinese breeds. The high number of haplotypes (22) for a sample size of 53 individuals indicates elevated genetic variability, contrasting with more homogeneous populations from other countries. This high diversity could be explained by the multiethnic origin of Cuban populations (González-Alonso *et al.*, 2025), which received influences from roosters brought from Spain, other regions of Europe, Asia, and Africa during the colonial period. The significant

departure from neutrality (negative Tajima's D) suggests that these populations may have experienced recent population expansions or directional selection processes (Al-Jumaili *et al*., 2020).

CONCLUSIONS

The sampled birds exhibited very high genetic variability. This study represents the first mtDNA-level genetic characterization of the Cuban Creole fighting rooster

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AUTHOR CONTRIBUTION STATEMENT

Research conception and design: AVG, GEGV, AAC, AVM, OUR, AAVP; data analysis and interpretation: AVG, GEGV, AAC, AVM; manuscript writing: AVG, GEGV, AAC, AVM, OUR, AAVP.

CONFLICT OF INTEREST STATEMENT

The authors state there are no conflicts of interest whatsoever.