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Study of the mitochondrial genome (*mtDNA*) of the Iraqi marsh buffalo

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Abstract

The study focused on examining a considerable part of the mitochondrial genome in the local buffalo of Al-Jubayish marshes to understand how much genetic variation exists within this population compared to buffalo breeds from other countries. Blood was collected from twenty animals, and the extracted DNA was amplified using a set of specific primers that cover several mtDNA genes. After confirming successful amplification, the samples were sent for sequencing, and the resulting FASTA files were analyzed through alignment, detection of variable sites, and the construction of a phylogenetic tree. A haplotype network was also prepared to show how the maternal lines are distributed among the animals. When the local sequences were compared with a larger set of global sequences, the results showed that the buffalo of Al-Jubayish have only five haplotypes—far fewer than those recorded in Indian, Egyptian, or Chinese buffalo. The analyses also revealed low values for both haplotype and nucleotide diversity, which means that the animals in this region are genetically quite similar to one another. The number of nucleotide differences was modest as well, supporting the idea of limited diversity in the local herd. Tajima's D value was close to zero, suggesting that the population has not undergone recent major changes or strong selective pressures.

Four variable sites were identified in the mtDNA fragment that was examined, and although the genotype frequencies differed slightly among individuals, the overall variation remained narrow. The haplotype network demonstrated that most Iraqi buffalo grouped into two main maternal clusters. One of these clusters included individuals that shared lineages with buffalo from India and Egypt, hinting at older historical connections. The other cluster appeared more specific to the Iraqi samples, likely shaped by geographic isolation and traditional breeding practices in the marshlands. Overall, the findings indicate that while the genetic diversity of local buffalo is lower than that seen in many global breeds, this stability may reflect long-term adaptation to the marsh environment and could be valuable in future conservation efforts.

Key words : mtDNA, Genetic diversity, Marshes buffalo

I. Introduction

Mitochondrial DNA (mtDNA) is often used in genetic and evolutionary research because it is passed from the mother only. This makes it easier to trace maternal ancestry without mixing from the father's side (Boore, 1999). Also, mtDNA changes faster than most nuclear genes, so researchers can use it to study differences between close populations or breeds. For this reason, mtDNA is considered useful in studies on domesticated animals, especially when researchers want to understand where a breed came from and how it adapted to its environment (Zhang et al., 2020). Buffaloes (*Bubalus bubalis*) are important livestock animals in many countries in Asia and the Middle East. They provide milk, meat, and are also used for work. They are generally divided into two types: river buffalo and swamp buffalo. River buffalo are mostly found in South Asia and the Middle East, while swamp buffalo are more common in Southeast Asia. Even though they share the same origin, each type developed different physical and adaptive traits because they lived in different environments for a long time (Yadav et al., 2022).The Iraqi marsh buffalo is a type of river buffalo and is

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mainly found in the Mesopotamian Marshes in southern Iraq. This region is known for high temperatures, high humidity, and changing water salinity. These conditions shaped the marsh buffalo, giving it good heat tolerance, strong dependence on water to cool its body, and feeding habits suited to marsh plants (Al-Aubaidi

& Mohammad, 2021). But in recent years, this animal has faced problems due to drying of the marshes and the decline of water resources.

Studying the mtDNA of the Iraqi marsh buffalo can help us understand where it originally came from and how it is related to other buffalo populations. This information is useful for conservation and for improving breeding programs. It also helps protect the genetic traits of this animal, which is considered part of the cultural and environmental heritage of southern Iraq.

II. Materials and Methods

The current study aimed to analyze the sequence of a large region of the mitochondrial genome (mtDNA) in local buffalo from the marshes of Al-Jubayish, located in the south of Nasiriyah city. The study included 20 buffalo heads (Bubalus bubalis). Blood samples were collected from the jugular vein of each animal, and DNA was extracted using a DNA extraction kit provided by Geneaid (Taiwan). Specific primers were used to amplify and analyze a targeted fragment of mtDNA, which included the Cytochrome c oxidase subunit III (*COX3*), NADH dehydrogenase subunit 3 (*ND3*), NADH dehydrogenase subunit 4L (ND4L), and NADH dehydrogenase subunit 4 (*ND4*). The primers B13-F and B13-R were used for amplification. Table (1) shows the amplified mtDNA region with a length of 2278 bp, as described by Parma et al. (2004), while Table (2) presents the PCR protocol used in this study.

Table(1) The primers used and the products size.

No.		Primer Sequence ^{3} to ^{5}	Product size.
1	**B13-F**	F- 5-GCT GCC TGA TAT TGA CAC TTG-3	2278 bp
2	**B13-R**	R- 5-GGG CTT CTA TTG TTA GAT TCA C-3	

Table (2) The protocol of PCR amplification

Step		Temp.	Time	Cycle
Initial Denaturation		95	5.00 min	1 time
	Denaturation	95	1 min.	**35 cycle**
	Annealing	55	1 min.	
	Extension	72	1.50 min.	
Final Extension		72	10.00 min	1 time

All successfully amplified samples were subsequently sent to Macrogen Company (Seoul, South Korea) for sequencing in order to identify nucleotide variations within the studied mtDNA region. The sequencing results were received in FASTA format and then aligned using BioEdit v7.2.5 software (Hall, 1999). Multiple sequence alignment was conducted to detect polymorphic sites and substitution patterns among the samples. To explore genetic relationships among individuals, a phylogenetic tree was constructed using MEGA v11 software. The UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm was applied, and evolutionary distances were calculated based on the nucleotide substitution model selected according to the

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best fit analysis (Tamura et al., 2021). haplotype networks were constructed using the Network version 10.2 software (Bandelt et al., 1999) to visualize genetic relationships among the studied sequences.

Furthermore, to visualize the maternal lineage distribution and assess haplotype diversity and other molecule parameters, a haplotype network was generated using Network v10.2 software. This analysis helped illustrate the evolutionary connections between haplotypes and provided insights into the genetic structure of the population under study.

III. Results & Discussion

The table (3) compares the data obtained from the local buffalo in the current study with reference mitochondrial sequences of buffalo recorded in the GenBank database (NCBI), in addition to comparing each sequence individually. This comparison aims to evaluate the degree of genetic diversity in the local buffalo population and to determine whether it shows similarities or differences in genetic variation compared to buffalo populations from different regions of the world. The DNAsp V.6 software was used to analyze molecular diversity parameters.

The total number of sequences included in the analysis was 120 (Table 3), of which 20 belonged to the local buffalo used in this study. The global reference sequences included 72 sequences from Indian buffalo, listed under the following GenBank accession numbers: MN756622, OR766351, OR766352, OR766353, OR766354, OR766355, OR766356, OR766357, OR766358, OR766359, OR766360, OR766362, OR766364, OR766365, OR766366, OR766367, OR766368, OR766369, OR766370, OR766371, OR766372, OR766373, OR766374, OR766375, OR766376, OR766377, OR766378, OR766381, OR766380, OR766382, OR766383, OR766384, OR766385, OR766387, OR766389, OR766392, OR766393, OR766394, OR766395, OR766396, OR766397, OR766398, OR766399, OR766400, OR766406, OR766407, OR766408, OR766409, OR766410, OR766411, OR766412, OR766417, OR766419, OR766420, OR766421, OR766422, OR766423, OR766424, OR766425, OR766426, OR766427, OR766429, OR766430, OR766431, OR766432, OR766433, OR766436, OR766437, and OR766440. Additionally, 25 sequences from Egyptian buffalo were included under the accession numbers: MT237604, MT237605, MT237606, MT237607, MT237608, MT237609, MT237610, MT237611, MT237612, MT237614, MT237615, MT237617, MT237619, MT237620, MT237622, MT237623, MT237624, MT237625, MT237626, MT237628, MT237629, MT237630, MT237631, MT237632, MT941070, MT942705, MT942706, and MT954916. Moreover, the dataset included two sequences from China (MN481528 and KX758295) and one sequence from the United States (NC_057438).

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Table (3) mtDNA sequences of local buffalo and reference copies from the NCBI GenBank

Group	country	Accession Numbers at NCBI
1	Iraq	20 sequences
2	India	72 sequences (MN756622, OR766351, OR766352, OR766353, OR766354, OR766355, OR766356, OR766357, OR766358, OR766359, OR766360, OR766362, OR766364, OR766365, OR766366, OR766367, OR766368, OR766369, OR766370, OR766371, OR766372, OR766373, OR766374, OR766375, OR766376, OR766377, OR766378, OR766381, OR766380, OR766382, OR766383, OR766384, OR766385, OR766387, OR766389, OR766392, OR766393, OR766394, OR766395, OR766396, OR766397, OR766398, OR766399, OR766400, OR766406, OR766407, OR766408, OR766409, OR766410, OR766411, OR766412, OR766417, OR766419, OR766420, OR766421, OR766422, OR766423, OR766424, OR766425, OR766426, OR766427, OR766429, OR766430, OR766431, OR766432, OR766433, OR766436, OR766437 & OR766440)
3	Egypt	25 sequences (MT237604, MT237605, MT237606, MT237607, MT237608, MT237609, MT237610, MT237611, MT237612, MT237614, MT237615, MT237617, MT237619, MT237620, MT237622, MT237623, MT237624, MT237625, MT237626, MT237628, MT237629, MT237630, MT237631, MT237632, MT941070, MT942705, MT942706 & MT954916)
3	China	2 sequence (MN481528 & KX758295)
4	USA	1 sequences (NC_057438)

Table (4) shows the molecular parameters for local buffalo exhibited only 5 haplotypes, compared to 28 in the global reference copies & the total to 32 haplotypes. This confirms the low genetic diversity in the local buffalo compared to global sequencing. The haplotype diversity value (HD) in the local samples was 0.626, lower than the HD value of 0.845 in global breeds. These lower values indicate less variation among the local buffalo and suggest a higher level of genetic homogeneity within the local herd. While the Nucleotide Diversity (Pi) value was recorded at 0.00043 for local buffalo compared to 0.00072 for global sequences, these low values indicate minimal genetic variation in local buffalo, reinforcing the observation of low genetic diversity among the studied local breed. The results also showed that the average number of nucleotide variations (k) for local sequences was 0.98421 compared to 1.63333 globally. This demonstrates that local buffalo have fewer variations in their genetic sequence compared to global buffalo. The Tajima's D value in local buffalo was -0.36786, while it was -2.02066 in global sequences. A negative value typically indicates natural selection or recent population growth.

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parameter	Buffalo		
	This study	**Reference (NCBI)**	**All**
Number of sequences	20	100	120
Number of variable sites	4	25	29
G+C content (%)	39.2%	39.2%	39.2%
Number of Haplotypes	5	28	32
Haplotype diversity(Hd)	0.626	0.845	0.870
Nucleotide diversity(Pi)	0.00043	0.00072	0.00086
Average number of nucleotide differences(k)	0.98421	1.63333	1.96807
Tajima's D	-0.36786	-2.02066	-1.93246

Table (4) shows the molecular parameters for local buffalo & Reference copies

The genetic value of local buffalo is closer to zero than that of global buffalo, meaning it is closer to a state of genetic equilibrium compared to global buffalo, which appears to have undergone greater population changes or selective breeding. In summary, based on the aforementioned findings, we can conclude that local buffalo possess less genetic diversity than buffalo in other parts of the world. This can be explained by buffalo rearing in a geographically limited environment with a heavy reliance on local resources and limited genetic exchange with external herds. Despite this lower diversity, it also indicates genetic stability resulting from long-term adaptation to the marsh environment, which is a significant advantage in genetic conservation programs.

The local buffalo population in the study showed only five haplotypes, compared to 28 haplotypes recorded in the global reference sequences, bringing the total to 32 haplotypes. This clear difference indicates reduced genetic diversity in the local buffalo relative to international populations (Zhang et al., 2020). The haplotype diversity (HD) in the local samples was 0.626, which is lower than the value of 0.845 observed in global breeds, reflecting a higher level of genetic similarity among individuals within the local herd (Ahmed et al., 2017). Similarly, the nucleotide diversity (Pi) was recorded at 0.00043 in the local buffalo compared to 0.00072 in global sequences, confirming the limited genetic variation in the local population. The average number of nucleotide differences (k) was also lower in the local samples (0.98421) than in global buffalo (1.63333), demonstrating fewer genetic substitutions in the mitochondrial genome of the local animals (Nassiry et al., 2009).

The Tajima's D value in local buffalo was -0.36786, while it was -2.02066 in global sequences. Negative Tajima's D values are typically associated with either recent population expansion or directional selection pressures. However, the value being closer to zero in the local population suggests a more stable genetic state, while the more negative value in global buffalo indicates greater demographic changes or selective breeding events (Parma et al., 2004).

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In general, these findings show that the local buffalo have lower genetic diversity compared to buffalo populations in other regions. This can be explained by breeding within a geographically restricted environment, limited gene flow from outside populations, and long-term adaptation to the marsh habitat of southern Iraq (Ahmed et al., 2017; Zhang et al., 2020). Despite the lower diversity, this condition also reflects a level of genetic stability, which may represent an advantage in conservation programs aimed at maintaining environmentally adaptive traits.

Table (5) shows the variable sites (Mutations) along the studied fragment of mtDNA of the local Iraqi buffalo.

No. of Mutation	Site of Mutation	Polymorphisms	No. of individual
1	1855.G>A	GG	18
		AA	2
2	1882.C>G	CC	18
		GG	2
3	2081.T>A	TT	16
		AA	4
4	2132.T>A	TT	17
		AA	3

Table (5) shows the positions of the four variable sites detected within the studied fragment of mtDNA. The first variable site is located at position 1855 (G>A), where the GG genotype was observed in 90% of the samples, while the AA genotype appeared in 10% of the individuals. The second variable site is at position 1882 (C>G), with the CC genotype occurring in 90% of the samples and the GG genotype in 10%. The third variable site occurs at position 2081 (T>A), where the TT genotype represented 80% of the samples and the AA genotype represented 20%. The fourth variable site is located at position 2132 (T>A), where the TT genotype was observed in 65% of the individuals, while the AA genotype appeared in 32% of the samples.

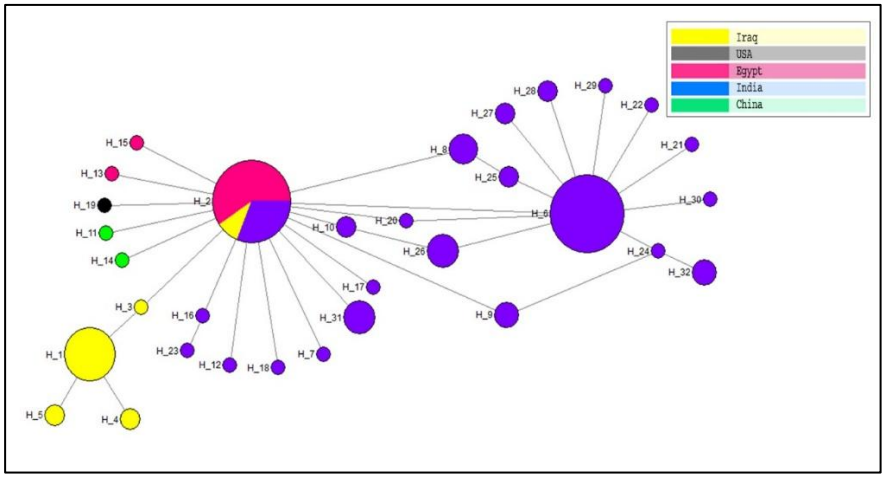

Figure (1) The haplotype network for mitochondrial DNA (mtDNA) for local buffalo and global reference copies

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The haplotype network presented in Figure (1) illustrates the genetic relationships among mitochondrial DNA sequences of Iraqi buffalo and reference populations from several regions, including India, Egypt, China, and the United States. Each circle in the network represents a unique haplotype, and the circle's size corresponds to the number of individuals sharing that same maternal lineage. Different colors are used simply to distinguish samples according to their geographical origins.

Most Iraqi buffalo samples cluster mainly within two haplotypes, H_1 and H_2. The H_2 haplotype includes individuals from Iraq as well as from India and Egypt, suggesting that these populations may share ancient maternal lineages. This pattern likely reflects historical movements of buffalo or the exchange of animals between regions. Such a relationship aligns with previous studies indicating that buffalo populations in the Middle East are genetically related to those in South Asia, where domestication is believed to have first occurred (Kumar et al., 2007; Lau et al., 2010).

Conversely, the H_1 haplotype appears more specific to Iraqi buffalo. This may point to the development of local maternal lines that have persisted across generations, particularly within the marshlands of southern Iraq. These regions have historically been relatively isolated, and herds were traditionally maintained within limited areas with minimal introduction of new animals from outside sources (Naderi et al., 2019). Such conditions could explain the presence of a distinct local genetic signature.

The greater number of haplotypes observed among Indian buffalo indicates high genetic diversity within those populations, supporting the view that India represents a major center for buffalo domestication and genetic variation. In contrast, the smaller number of haplotypes in the reference groups from Egypt, China, and the United States likely reflects limited sample sizes or lower within-population diversity (Zhang et al., 2020).

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