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Research Article

D-LOOP REGION SEQUENCE ANALYSIS OF MITOCHONDRIAL DNA IN IRAQI MARSHES BUFFALO

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Abstract

The origins of the domestic water buffalo remain contentious, but with the development of modern technologies it has become possible to predict the origin of local buffalo breeds found in different regions of the world, and several studies were used to reveal the complete genome sequence of mitochondrial mtDNA or use one of the mtDNA genes. We chose the D-loop region with a size of 452 bp using the Sequencing technique. Samples were used for 15 buffaloes from the marshes of southern Iraq, specifically from the Hammar region of ThiQar Governorate. in addition to were collected 115 reference copies of the nucleotide sequences of the D-loop regions from different countries, including China, India, Pakistan, Egypt, USA and Germany, and a set of Bioinformatics software were used to analyze genotypes and haplotypes data and their distribution, in addition to neutrality tests, as well as drawing a network of Haplotypes and Phylogenic tree. The results indicated that less genetic diversity than in the Indian, China, and Pakistani buffalo. Where the results of the analysis of nucleotide sequences for the D-loop region in mtDNA gave four haplotypes of relationships and most of the samples of this study shared one haplotype with the Chinese buffalo. Pakistani buffalo, and the results showed that the sequences collected from the GenBank have high genetic diversity in the D-loop region, and it may be due to the fact that this part of the mtDNA is not coding for a protein. The nucleotide sequences of the D-loop region were recorded for all study samples of local buffaloes in the GenBank under accession numbers LC729299 up tpLC729313.

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1. Introduction

The Asian buffalo is classified into two types: the swamp buffalo (*Bubalus carabanesis*), found in eastern Asia, and the river buffalo (*Bubalus bubalis*), which is found in the western half of Asia such as Iraq, as well as Egypt and Europe outside Asia. *Bubalus bubalis* is usually large in size, black or gray Dark with curved horns, weights range about 300-1000 kg. (Mudar and Anderson 2007). It is believed that during the medieval period buffalo domestication occurred in the regions of the Indian sub-continent during the medieval period (Zhang, 1987). River buffaloes have adapted well to swamps and flood-prone areas, so we find their presence most in Iraq in the marshes and swamps on the sides of the rivers, as well as the tidal plains in Australia (Darwin) as well as the Amazon in America (Al-Obaidi *et al*., 2007). Evaluation of genetic variability and genetic association in populations is essential to control the loss of genetic diversity for selective

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breeding, In the same way, genomic tools will allow for reproduction and improve production. Genetic diversity in livestock is also very useful in distinguishing between populations. To discover genetic differences between populations, as this depends on phenotypic traits or the use of genetic diversity methods (Mastrochirico Filho *et al*., 2019; Taberlet *et al*., 2011).

To identify variations in the maternal lineage, the Mitochondrial genomes (mtDNA) are usually explored. In the Mitochondrial genome, there are Cytochrome b (Cyt B) D-loop region and Cytochrome Oxidase I (COI) gene markers that are often used to in determining genetic variance (Kumar *et al*., 2007; Saputra *et al.*, 2013; Paraguas *et al.*, 2018). Previous studies of mtDNA variation using RFLP Technique restriction fragment length polymorphism (Amano *et al.*, 1994; Tanaka *et al.*, 1995) have shown genetic variations between the types, and have considered their origin, divergence and domestication. The sequencing method of genes was used to analyze the sequence of cytochrome b and 16S rRNA genes in revealing the genetic diversity of the Iraqi local buffalo (Hamad *et al*., 2021).

We present here results for DNA sequence variation in mtDNA control region (D-loop). The D-loop is the most variable portion of the mammalian, and is commonly variable at the intraspecific level, making it useful for studies of genetic variability among populations, phylogenetic analysis and Haplotypes analysis. Thus our aims are to compare D-loop variation, particularly among swamp type populations of marsh buffaloes in southern Iraq and a comparison of the genotypes they carry compared to the reference copies recorded in the Genebank.

2. Materials and Methods

Fifteen local buffaloes were used from the Hmmar marsh area in Thi-Qar city. Blood samples were collected from the animals from the jugular vein. DNA extraction, Polymerase Chain Reaction (PCR) amplification and Electrophoresis was performed on a Biology Laboratory at the Marsh Research Center, Thi-Qar University, Amplification of a 452 bp of the selected fragment of the D-loop region, The primers with amplification of D-loop region the following nucleotide sequences: Forward, 5' ATCCCTCTTCTCGCTCCG -3'and Reveres, 5' TATGTCCTGTGACCATTGACTG -3'. Mixture of PCR was 25 µl, containing 12 µl Master Mix, 1 µl for each primer forward & reverse, 3 µl DNA template Concentration 30 - 60 ng and 8 µl dd water. Program of PCR was 95 °C for 5 min for initial denaturation, followed by 35 cycles of 95 °C for 1 min, 58 °C for 45 sec., 72 °C for 1 min and a final extension at 72 °C for 7 min. The PCR products were migrated method by electrophoresis on a 1, 5 % agarose gel in parallel with a 100 bp DNA marker. The sequencing of PCR products with D-loop region was obtained by MACROGEN company. Nucleotide diversity and Haplotype diversity (HD) and were calculated, and the results of Tajima and Fu test were also analyzed using DNAsp V 5.10 (Librado and Rozas, 2009). The haplotypes network was drawn according to the Median Joining (MJ) algorithm using Network program. V 5.0.1.1 17 Bandelt *et al*. (1999). The phylogenetic tree was drawn using Mega 7 V.0.26 program (Kumar *et al*., 2016).

3. Results and Discussion

After completing the multiple alignment analysis using Bioedit V. 7.2.6 (Hall, 1999), the results indicated that there were three change sites between the 15 sequences only, and the ratio of nitrogenous bases (G + C) constituted 37 % of the total sequence. The three mutations detected among all local samples formed four haplotypes (H1, H2, H3 and H4). As shown in figure No. (1) and the figure for Haplotype diversity (Hd) the value of 0.543. Variance of Haplotype diversity was 0.01761, the nucleotide diversity was the result of 0.133 (Table - 1). For the polymorphisms data, nucleotide and Haplotypes diversity of the D-loop region of the animals in this study only.

Table – 1: Polymorphisms data for D-loop region for the Iraqi local buffalo

Polymorphisms data	Value
Number of sequences	15
Number of variable sites	3
Total number of mutations	3
G+C content	0.37
Number of Haplotypes	4
Haplotype diversity (Hd)	0.543
Variance of Haplotype diversity	0.01761
Nucleotide diversity (per site) (Pi)	0.133
Average number of nucleotide differences (k)	0.610

Totally, 115 reference copies of the D-loop nucleotide sequences were collected from different countries (India, China, Pakistan, Egypt, USA and Germany) in addition to 15 samples of the Iraqi local buffalo, which were recorded with independent accession numbers for our study for the first time starting from the number LC00000 up to LC000001 Upon completion of the multiple alignment analysis, an analysis was performed as measures of genetic diversity for the D-loop sequences of our local animals with reference copies from the gene bank as indicated above in Table - 2. The Table - 3 refers to Polymorphisms data for D-loop region for the Iraqi local buffalo & reference copies of the Genebank. The sum of the compared sequences for the D-loop region was 130 sequences. The total number of mutations that resulted when conducting multiple alignment analysis was 213 mutations, thus forming the nucleotide diversity value of 0.01736. The results showed that the value of the Haplotype diversity (Hd) was recorded at 0.910 ± 0.00016, and it is clear from this value that the buffalo shows high genetic diversity, especially in the global strains taken from the Genebank. Non-encoded regions are high (Nisan, 2014). The value of Haplotype diversity (Hd) for the D-loop region in our local animals were recorded as 0.543 compared to 0.888, 0.00998, 0.00080 and 0.03247 for the countries of India, Egypt, China and Germany, respectively (Table - 4). The difference in HD values may be to the difference in the number of comparative sequences for each country or due to the mixing that occurs between the breeds in different countries. When we return to Table - 3, we note that the G + C content amounted to 37 %, which is the same percentage for the sequences of our local animals. As for the value of the Nucleotide diversity (per site) (Pi) scored 0.01736 for all strains compared to 0.133 for our animals in the current study only.

Table – 2: Accession numbers for the reference copies of the Genebank as well as the copies recorded for this study from the D-loop region

No.	Country	Accession Numbers	Gene
1	Iraq	LC729299, LC729300, LC729301, LC729302, LC729303, LC729304, LC729305, LC729306 LC729307, LC729308, LC729309, LC729310, LC729311, LC729312, LC729313	D-loop
2	China	KR008855, KR008550 , GQ260327, KR008100, MN756622, KR008058, KR008012, EU268909, KR008017, MN481528, MK415619, MK415614, MK415613, MK415608, KR008101, KR008039, KR007980, MW013500, GQ260333,GQ260326,	D-loop
3	Egypt	MT237631, MT237629, MT237628, MT237627, MT237624, MT237623, MT237621, MT237620, MT237619, MT237611, MT237610, MT237609, MT237607, EU268908,EU268907, EU268905, EF536326,	D-loop
4	India	EF464456, EF464453, EF464447, EF464441 EF464439, EF464437, EF464435, EF464433, EF464429, EF464443, AF475235, AF475241, EF464331, EF464332, EF464364, EF464372, EF464386, EF464408, EF464438,EF464450, MT942706, EF464454, MT942706, MK415618, MK415615, MK415612, EF464455, EF464424, EF464394, EF464367, EF464431, EF464400, EF464398, EF464389, EF464377, EF464358, EF464357, AF475273, AF475244, AF475240, AF475223, AF475211, AF475199, AF475192, MK415606, EF464361, EF464348, EF464336, EF464333, EF464327, AF475190, AF475258, MT009487, MT954916,	D-loop
5	Germany	AF197217, AF197215, AF197203, AF197200, AF197198, AF197197, AF197196, GQ260332,	D-loop
6	USA	NC_057438, NC_049568	D-loop
7	Pakistan	KM453749, KM453748, KM453747	D-loop

Table – 3: Polymorphisms data for D-loop region for the Iraqi local buffalo and reference copies of the Genebank

Polymorphisms data	Value
Number of sequences	130
Number of variable sites	199 site
Total number of mutations	213
G+C content	37%
Number of Haplotypes	34
Haplotype diversity (Hd)	0.910
Variance of Haplotype diversity	0.00016
Nucleotide diversity (per site) (Pi)	0.01736
Average number of nucleotide differences (k)	7.22242

Table – 4: Polymorphisms data for D-loop region for the Iraqi local buffalo & reference copies of the Genebank

Country	Haplotype diversity (Hd) ± Variance of Haplotype diversity	Number of sequences
Iraq	0.543 ± 0.01761	15
India	0.888 ± 0.00059	55
Egypt	0.708 ± 0.00998	19
China	0.938 ± 0.00080	21
Germany	0.250 ± 0.03247	8
All country	0.910 ± 0.00016	130

The results of the analysis of neutrality tests showed for D-loop region for the Iraqi local buffalo and reference copies of the Genebank Table – 5. The value of the Tajima's D value was 2.67450 with a highly statistically significant (**, $P < 0.001$). The value of Fu and Li's D* test was recorded as a negative value of -10.77407, with a statistically significant significance (**, $P < 0.02$), as for the value of Fu and Li's F It also gave a negative value of -8.49789 with a statistically significant (**, $P < 0.02$).

It is believed that the negative values of most neutrality tests except for Tajima's D test are due to the expansion in the size of buffalo breeds and populations in the world and the spread of buffalo within the region across a wider geographical range, because neutrality tests used to measure the probability that a population had undergone demographic challenges such as genetic drift or expansion in population size (Fu, 1997).

Table - 5: Neutrality Tests for D-loop region for the Iraqi local buffalo & reference copies of the Genebank

Neutrality Tests	Value	Statistical significance
Tajima's D	2.67450	***, P < 0.001
Fu and Li's D* test statistic	-10.77407	**, P < 0.02
Fu and Li's F* test statistic	-8.49789	**, P < 0.02
Fu's Fs statistic	-4.317	

The results of the Haplotypes Network showed a Figure - 1 for the D-loop region for all the local and global sequences collected by the Genebank, as we indicated in Table - 3 for the river buffalo *Bubalus bubalis*, which constitutes 34 haplotypes, four of which were for the local buffalo in this study. The Haplotypes H1 consistence from the local buffalo, Chinese and Pakistani buffaloes. As for the remaining three Haplotypes from local Iraqi buffalo. The American buffalo also took independent Haplotypes that H33 and H34. The German buffalo also took independent Haplotypes, which are H31 and 32, and among the non-independent Haplotypes that participated It has a group of countries such as the patterns H12, H19 and H20, where the countries of China, India and Egypt participated in it, and the Haplotypes H6, H13, H15 constituted from the countries of China and India. most of the Indian buffalo breeds were distributed with a greater number of Haplotypes independently such as H7, H8, H10, H11, H17, H18, H19, H28 and H29. It also gives two independent Haplotypes of the Egyptian buffalo, which are H25 and H27. Sometimes the Haplotypes that are close to each other, which arise from the same branch with their participation in almost the same mutations, and by combining these Haplotypes form a clear network of them called the Haplogroup, as we see clearly in the Haplotypes that include the Iraqi local buffalo in this study with The Chinese buffalo and the Pakistani buffalo have the Haplotypes (H1, H2, H3, H4 and H5) and a second Haplogroup in Figure – 1, H12, H18, H22 and H28, which is shared by the Indian, Chinese and Egyptian buffaloes. We note from the above results that there is a wide diversity of Haplotypes due to the presence of changes in several nucleotide sites for the D-loop region of different countries of the river buffalo, and this may be due to the expansion in the size of buffalo breeds and populations in the world and the spread of buffalo within the region across a wider geographical range, because neutrality tests used to measure the probability that a population had undergone demographic challenges such as genetic drift or expansion in population size (Fu, 1997).

The results of the Phylogenic Tree analysis were shown using Mega V 7.0.26 program (Kumar *et al*., 2016), where the tree was drawn using the Neighbor-Joining tree (NJT) method based on D-loop region. The tree was reconstructed using the 130 reference copies of *Bubalus bubalis* identified in our alignment of 431 base pair. The Iraqi buffalo had taken a main independent branch jointly with the Chinese and Pakistani buffaloes, and some members of the local buffalo had independent branches with secondary branches. In the main branches, many Chinese, Indian and Egyptian buffalo participated together, While the American buffalo and the German buffalo rode with reclining secondary branches (Figure - 2). The nucleotide sequences of the D-loop region were recorded for all study samples of local buffaloes in the GenBank under accession numbers LC729299up tpLC729313.

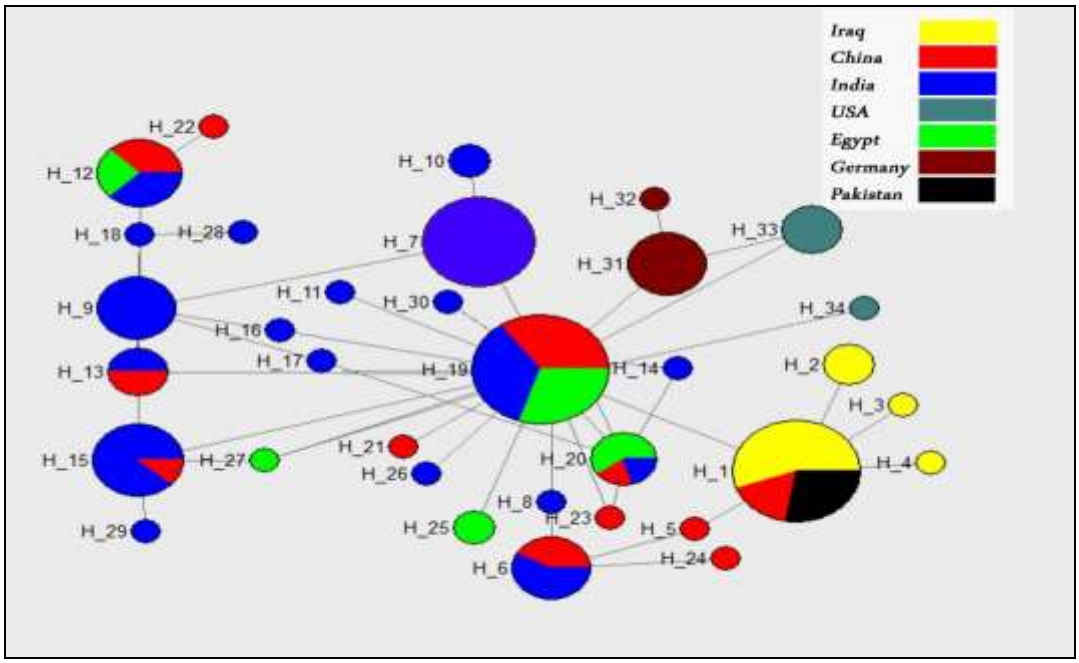

Figure – 1: Haplotypes Networkfor D-loop region for the Iraqi local buffalo & Reference copies of the Genebank

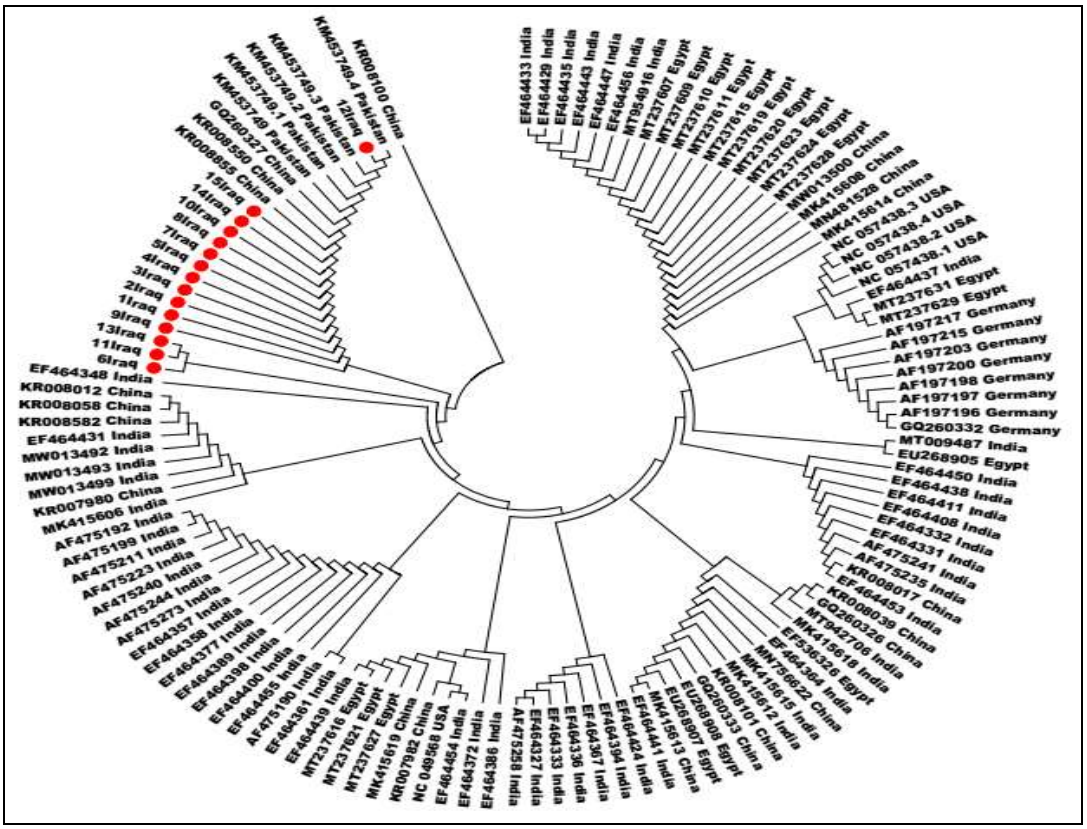

Figure – 2: Phylogenic Treefor D-loop region for the Iraqi local buffalo &reference copies of the Genebank

4. Conclusion

The genetic diversity of the Iraqi marsh buffalo in the Hammar region of ThiQar governorate showed less genetic diversity than in the Indian, China, Pakistani buffalo. Where the results of the analysis of nucleotide sequences for the D-loop region in mtDNA gave four haplotypes of relationships, and most of the samples of this study shared one haplotype with the Chinese buffalo. and the Pakistani buffalo, and the results showed that the sequences collected from the genebank have high genetic diversity in the D-loop region, and it may be due to the fact that this part of the mtDNA is not coding for a protein. The study needs to take wider areas of mtDNA, including whole genome of mitochondria (mtDNA), in addition to including wider populations of the local Iraqi buffalo in the study and more areas, especially in southern Iraq, where it is abundant, especially in the southern and central marshes in Iraq.

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