International Journal of Future Engineering Innovations

Haplotype Diversity to *SPEF2* Gene in the Iraqi Holstein Bulls and Iraqi Buffalo Male

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Article Info

ISSN (online): 3049-1215
Volume: 02
Issue: 06
November - December 2025
Received: 13-10-2025
Accepted: 15-11-2025
Published: 11-12-2025
Page No: 44-50

Abstract
SPEF2 gene represents one of the important genes that are directly related to the fertility of farm animals, because of its work in the production of a protein that participates in the normal growth of the tail of the sperm and thus has a direct effect on the motility of the sperm. This research seeks to uncover the haplotype of the *SPEF2* gene in both Holstein Iraqi bulls and male Iraqi buffaloes. 7 bulls and 7 male buffaloes were used, belonging to the artificial insemination centre in Abu Ghraib (fertility information) between the ages of 2-3 years for the period from 10/11/2022 to 10/2/2023. All animals were under veterinary supervision. By using a synthetic vagina, semen had been collected, then DNA was obtained, PCR amplification was performed, after that, after purifying the product of PCR, the sequence analysis was performed. Some bioinformatics analyzes were conducted to detect possible haplotypes of *SPEF2* gene in each of buffalo cattle. Two haplotypes were obtained in each of the cattle and buffaloes, this diversity in haplotypes can explain the genetic diversity of this gene in farm animals in Iraq and can contribute to the selection of animals with high fertility, as this gene can be considered as a molecular marker for the selection of fertile males.

DOI: https://doi.org/10.54660/IJFEI.2025.2.6.44-50

Keywords: *SPEF2* Gene, Iraqi Holstein Bulls, Iraqi Buffalo Male, Gene Diversity, Semen Characteristics

Introduction

Animal production in Iraq is an essential sector of the country's economy, yet this sector faces numerous issues (Al-Salihi, 2012). Due to a variety of factors, such as climate circumstances and the absence of effective scientific programs for selecting animals with high production capacities, (Al-Haboby *et al*., 2014) [3] Climate change resulted in significantly higher temperatures, which had a negative impact on farm animals' fertility, particularly on the quality of bulls' semen. (Alejandro *et al*., 2014) [2]. Iraqi Holstein bulls are important in the sustainability of livestock, so studies are still continuing to try to develop their production and raise fertility (Mohammed *et al*., 2020) [10], as for the Iraqi buffalo, despite its economic importance, it suffers from many different problems, which somewhat led to a decrease in its products for various reasons, the most important of which may be the lack of recent studies that dealt with it (Rossi *et al*., 2018) [26]. The ability of animals to have new offspring is referred to as fertility. (Utt., 2016) [36]. In ruminants, fertility is very important because it directly affects the different production traits and thus has a significant impact on the livestock industry (Abdollahi-Arpanahi *et al*., 2017) [1]. Most workers in the livestock industry in the world suffer from fertility problems, for example, fertility decline causes economic losses estimated at about one billion dollars in the livestock industry each year in the United States (Prevatt *et al*., 2018) [23]. On the other hand, in the United States of America alone, 33% of cows in herds raised for meat production are excluded annually due to low fertility or infertility (Moorey and Biase, 2020) [11], therefore, the fertility of bulls is an important economic characteristic in sustainable livestock production. Although bulls produce large amounts of semen, some bulls may still have poor reproductive issues, which has a substantial negative financial impact on the livestock business. (Ugur *et al*., 2022) [34].

As a result of the development of modern technologies and the development of science, it has become necessary to search for new methods for selecting animals with higher efficiency. One of these techniques is the selection of animals on the basis of molecular markers (Qin *et al*., 2017) [24], a molecular marker is defined as any stable, inherited change that can be measured or detected in an appropriate way and that can subsequently be used to determine whether a specific phenotype or genotype is present. (Reshma and Das, 2021) [25]. Numerous studies have indicated that there are many genes that can be molecular markers in semen (Habib *et al*., 2017; [12] Habib *et al*., 2018) [13]. Modiba *et al*., (2022) [21] indicated that the *SPEF2* gene is one among the potential genes that influence the traits of cattle semen directly, therefore it can be considered as one of the molecular markers in the selection of cattle males.

The *SPEF2* gene is known as sperm flagella 2, it may also be called KPL2 according to the protein it produces, this gene is very necessary for the normal growth of the sperm tail, which directly affects sperm motility, and therefore it has a very important effect on semen fertility (Guo *et al*., 2014) [17]. The *SPEF2* gene is a multi-exon gene in cattle, it contains 35 introns and 36 exons (Sironen *et al*., 2010) [32], while another study indicated that this gene consists of 42 exons in both cattle and buffaloes, in cattle, it is located on chromosome 20 while in buffaloes it is located on chromosome 19 (Davis *et al*., 2023) [6]. Because of how crucial this gene is for the growth and development of the sperm tail, therefore, the occurrence of mutations in it can affect the formation of sperm, and thus its motility may be affected (Sironen *et al*., 2006) [31].

Due to the absence of previous studies that dealt with this gene in detail in Iraq and the lack of studies globally, we sought to study the diversity of the haplotype to the *SPEF2* gene in the semen of both Iraqi Holstein bulls and Iraqi male buffaloes.

Methods and Materials

In the Department of Animal Production, College of Agriculture, University of Basrah, Basrah, Iraq, this study has been conducted (longitude 47.7433690, latitude 30.5627250 north of Basrah). 7 bulls and 7 male buffaloes (Known fertility) between the ages of 2-3 years, were used, belonging to the artificial insemination centre in Abu Ghraib, Animal Resources Department, Iraqi Ministry of Agriculture (Longitude 1922070.44, Latitude 3095550.33 northwest of Baghdad) for the period from 10/11/2022 to 10/2/2023. All animals were in good health, free of disease, and under constant veterinary supervision. On the other hand, Semen was obtained by the use of a synthetic vagina.

The Extraction of DNA

DNA isolated using the technique described by Loffler *et al*., (2021) [20], using the (chelex 100) kit and according to the procedure the manufacturer advises. Using a Nanodrop, the concentration and purity of DNA were calculated.

The Amplification of PCR

The amplification reaction was adopted with a volume of 25 µl for both bulls and buffalo's male as it consisted of 9.5µl water nuclease-free, 12.5 µl of 2X PCR master mixes, 1.0 µl (10 µM) of each primer (Forward and reverse),1.0 µl (75 ng) DNA template. The primer in bulls was F-CAGGCGGATTTCAACTCCCT, R-CCTCTCACCGAGCCACTTTT, while in buffalo's male was F- TAGTAGTAGCGCTGCCTTGG, R-TGGAAGATTCATAGCCACTCCTG (Kõressaar *et al*., 2018) [18]. The PCR condition for bulls and buffaloes was 98 °C (initial denaturation) for 5 min pursued by 1 cycle, after that, 35 cycles of denaturation for 15 sec at 98º C, then the annealing was done for 30 sec at 55° C, the extension was 30 sec at 72 °C, the final extension was carried out for 5 min at ambient 72°C then pursued by 1 cycle. A 15% ethidium bromide stain was utilised (0.5 µg/ml agarose gel) to detect the PCR product, 1 Kb DNA ladder was used, the purification was done by following the manufacturer's instructions when using a Qiagen kit (Germany).

The Analysis of Sequences

Sequence analysis has been completed in Malaysia's first BASE Laboratory, and the BLAST analysis and the Multiple Sequence Alignment have been done (Boratyn *et al*., 2019) [7]. The results of the Sequence were compared with the highest match rate in the NCBI to detect the possible molecular change (The comparison was in cattle with accession number KF733182, which represents Bos taurus in China, while the comparison was in buffalo with accession number XM 025270630, which represents Bubalus bubalis in India). To uncover the 3D structure of protein the Swiss model (Waterhouse *et al* 2018) [37] has been used, Mega software was used to analyse the phylogenetic tree (Tamura *et al*., 2021) [33].

The Results and Discussion

The outcomes of the DNA examination revealed that the ratio of 260/280 was close to 1.8 for all study samples, this is consistent with what both Habib *et al*., (2022) [14,15] and Hindash and Hindash, (2022) [16] indicated, as this ratio is ideal for DNA amplification.

The size of the DNA product in bulls was 5338 bp and in buffaloes 5572 bp, this is consistent with Nikitkina *et al*., (2021) [22] and Deng *et al*., (2016) [9] respectively (Fig 1,

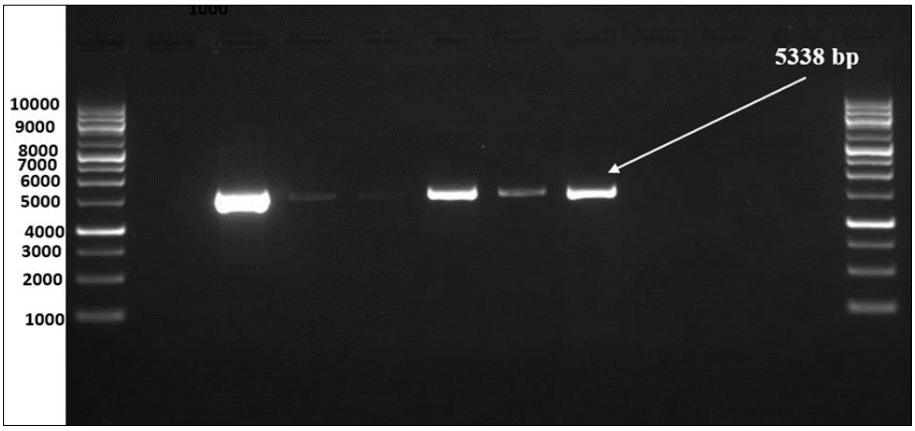

Fig 1: Gel Electrophoresis of PCR product of SPEF2 gene in Cattle

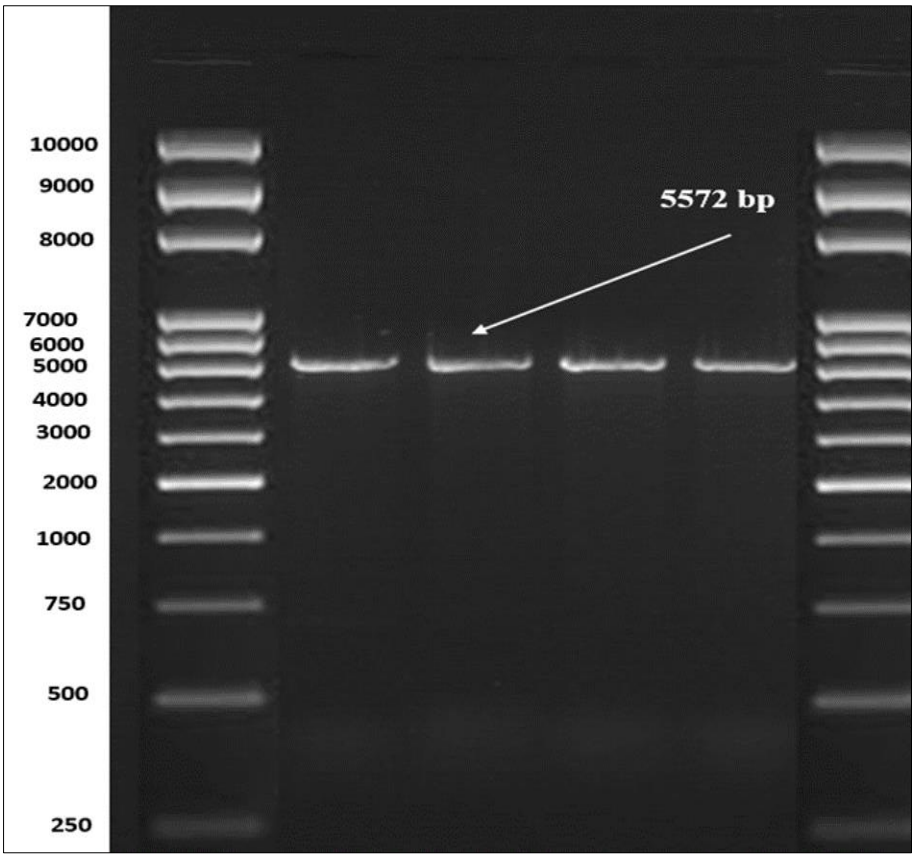

Fig 2: Gel Electrophoresis of PC R product of SPEF2 gene in Cattle

The results indicate that there are two haplotypes in both cattle and buffaloes due to the occurrence of a number of different mutations (These mutations are summarized in Tables 1 and 2, respectively). It is important to note that a number of mutations were involved in both haplotypes, whether in cattle or buffalo (they have not been previously recorded anywhere), perhaps the reason is that these animals are of common origin (Habib, 2020) [14,15]. The frequency of silent mutations was lower in all the animals under study, and although they do not code for a new amino acid, their effect could be phenotypic (Ujjan *et al*., 2022) [35], these mutations may also affect gene expression by changing its levels, It may affect mRNA stability by abnormal splicing (Lech, 2022) [19]. A number of missense mutations occurred in all haplotypes obtained in the study, the impact of these mutations relies on how each new and old amino acid differs from the others. (Ujjan *et al*., 2022) [35], Moreover, this variation within the gene (due to different mutations) could be a sign of different fertility among the animals studied (Sinha *et al*., 2022) [29]. The presence of mutations, whether in cattle or buffaloes, may indicate the presence of genetic variation in these animals that may contribute effectively to selection (Samuel *et al*., 2023) [28].

Three-dimensional protein analysis in both cattle and buffaloes revealed a clear difference in the protein profile of each haplotype due to mutations (Fig 3 A, B and 4 A, B respectively***)***, silent mutations, although they do not change the amino acid, alter the protein's structure, which could have an impact on how stable and functional the protein is (Bali and Bebok, 2015), on the other hand, missense mutations may contribute to changing some protein properties according to the new amino acid, which can have a greater impact on its stability and functionality (Chen *et al*., 2022 [8].

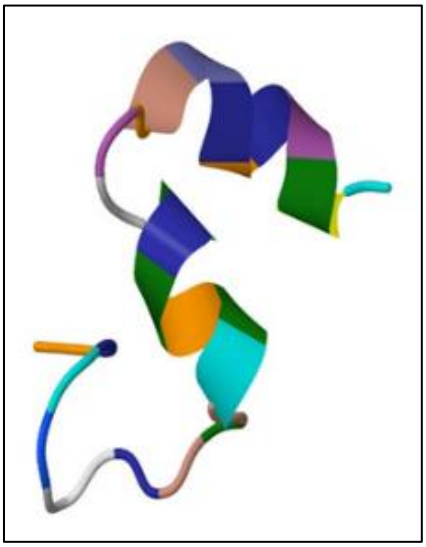

Fig 3A: 3D structure of protein haplotype 1

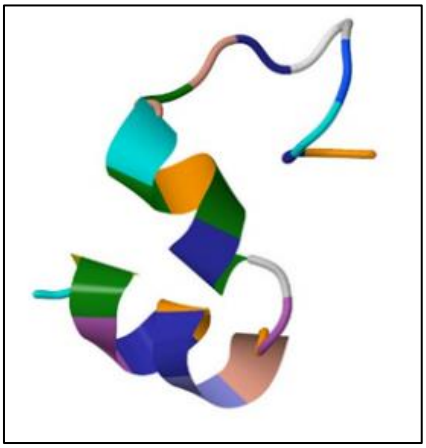

Fig 3B: 3D structure of protein haplotype 2 Cattle

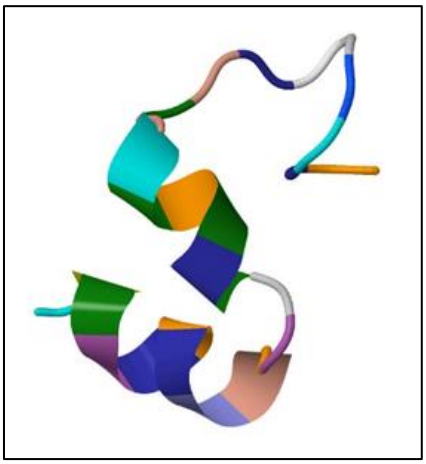

Fig 4A: 3D structure of protein haplotype 2 Buffalo

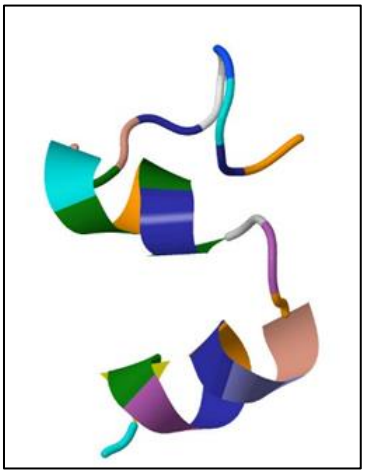

Fig 4B: 3D structure of protein haplotype 2 Buffalo

Table 1: Mutations in the *SPEF2* gene in Iraqi Holstein bulls

	Number of mutations	Nucleotide in citation Gene KF733182	Nucleotide in Haplotype	Site	Mutations	
					Type of mutation	The change in amino acids
Haplotype 1 (LC754316)	1	G	C	13	Missense	Glutamic Acid change to Glutamine
	2	G	T	75	Silent	
	3	A	T	634	Missense	Lysine change to Serine
	4	A	C	635	Missense	Lysine change to Serine
	5	A	C	687	Missense	Lysine change to Asparagine
	6	A	G	795	Silent	
	7	C	A	1130	Missense	Proline change to Histidine
	8	T	A	1994	Missense	Isoleucine change to Asparagine
	9	C	T	5269	Silent	
Haplotype 2 (LC754317)	1	G	C	13	Missense	Glutamic Acid change to Glutamine
	2	A	T	634	Missense	Lysine change to Serine
	3	A	C	635	Missense	Lysine change to Serine
	4	A	G	795	Silent	
	5	A	T	797	Missense	Asparagine change to Lysine
	6	C	G	1129	Missense	Proline change to Aspartic Acid
	7	C	A	1130	Missense	Proline change to Histidine
	8	T	C	1131	Missense	Proline change to Histidine
	9	A	C	4398	Silent	
	10	A	T	5111	Missense	Lysine change to Methionine

Table 2: Mutations in the *SPEF2* gene in Iraqi Buffalo

	Number of mutations	Nucleotide in citation Gene	Nucleotide in Haplotype	Site	Mutations	
					Type of mutation	The change in amino acids
Haplotype 1 (LC754318)	1	T	A	193	Missense	Phenylalanine change to isoleucine
	2	G	A	892	Missense	Glycine change to arginine
	3	T	G	1706	Missense	Phenylalanine change to cysteine
	4	A	T	2058	Silent	
	5	G	A	3571	Missense	glycine change to serine
	6	A	T	3667	Missense	Threonine change to serine
	7	G	T	3927	Silent	
Haplotype 2 (LC754319)	1	C	T	23	Missense	Proline change to leucine
	2	T	A	193	Missense	phenylalanine change to isoleucine
	3	T	A	208	Missense	Tryptophan change to arginine
	4	C	G	254	Missense	Proline change to arginine
	5	G	C	1296	Missense	Leucine change to phenylalanine
	6	G	C	1918	Missense	Glycine change to arginine
	7	T	G	2043	Missense	phenylalanine change to leucine
	8	A	T	2058	Silent	
	9	G	A	2096	Missense	Arginine change to lysine
	10	G	A	2114	Missense	Arginine change to lysine
	11	G	T	3927	Silent	
	12	A	G	3963	Silent	

The results of the phylogenetic tree analysis (Fig 5) indicate the clear variation in the haplotypes obtained in the study, It is entirely compatible with the other findings of the present investigation, the bulls were closer to the Chinese Bos taurus (according to the analysis of the studied gene), this indicates the great spread of this breed, the buffalo was closer to the

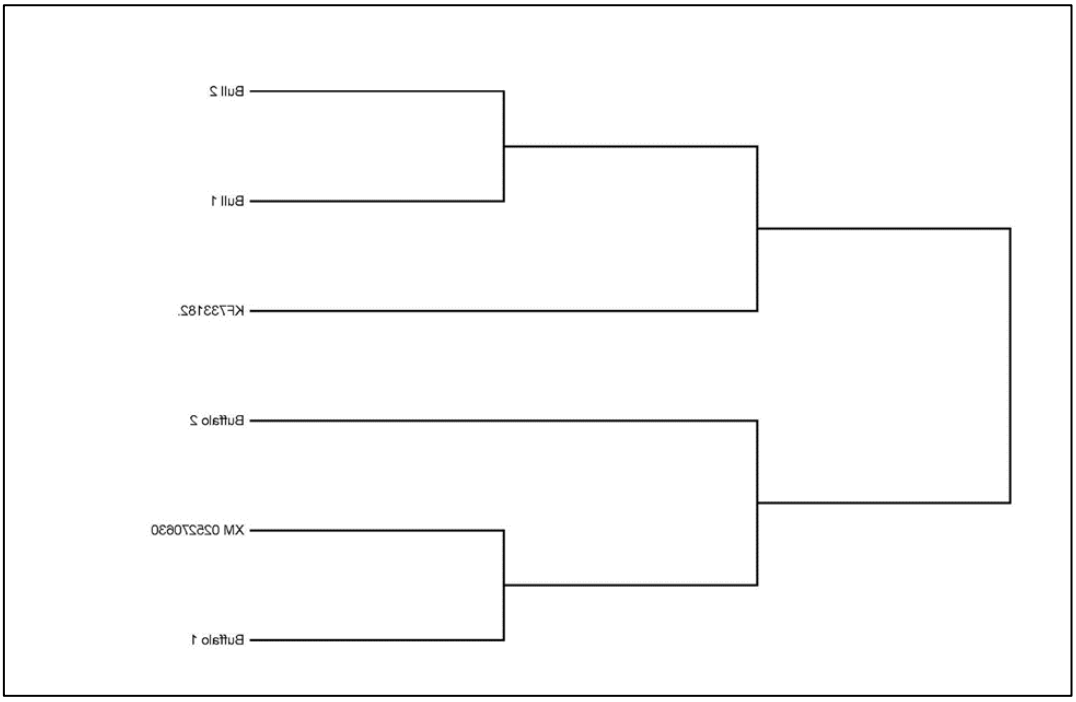

Fig 5: phylogenetic tree of *SPEF2* gene in Iraqi Holstein bulls and Iraqi buffaloes male

Indian breed, this indicates the close genetic relationship of the current study animals with these origins and their participation in common ancestors (Samuel *et al*., 2022) [27], Moreover, the lack of research that looked at the *SPEF2* gene, providing no additional data for comparison, may be to blame for the results of this study converging with both cattle in China and buffaloes in India.

Conclusion

The findings of this study show that the *SPEF2* gene is genetically diverse in Iraqi cattle and buffaloes, this diversity can contribute to enhancing the effectiveness of the gene, especially its direct effect on fertility, and therefore it can be a molecular marker for selecting the most fertile males. Therefore, there is a need for more studies that deal with this gene and link it to different fertility criteria, such as semen characteristics.

Acknowledgments

We want All employees of the artificial insemination center for their assistance in the completion of this research.

Ethical considerations

Data for this research was collected without handling or disturbing animals.

Conflict of Interest

The authors declare no conflict of interest.

Funding

This research is part of a master's thesis for the first researcher and a scholarship to study a master's study, at the University of Basra, College of Agriculture, Department of Animal Production.

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How to Cite This Article
Salem OB, Habib HN, Mohammed OA. Haplotype diversity to SPEF2 gene in the Iraqi Holstein bulls and Iraqi buffalo male. Int J Future Eng Innov. 2025;2(6):44–50. doi:10.54660/IJFEI.2025.2.6.44-50.