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Turki & Al-Anbari
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ACTN3/ EXON-19 GENE POLYMORPHISM AND ASSOCIATION WITH THOROUGHBRED HORSES PERFORMANCE IN IRAQ

Turki, W. Z. [1*] ***, Al-Anbari, N. N.*** [2]
[1]Department of Animal Production, College of Agriculture Engineering Sciences, University of Baghdad, Baghdad, Iraq. waleed.zaien1101a@coagri.uobaghdad.edu.iq
[2]Professor, Department of Animal Production, College of Agriculture Engineering Sciences, University of Baghdad, Baghdad, Iraq. nassr.n@coagri.uobaghdad.edu.iq

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ABSTRACT

The research was conducted in the Iraqi Equestrian Club (15 km west of Baghdad city center) for the period from 10/1/2021 to 11/30/2022, with the aim of revealing the ACTN3 gene/ EXON-19 region polymorphism and its relationship to the performance of Thoroughbred horses. British in Iraq. It was found that there are 3 variants (3 SNPs) in the ACTN3 gene with respect to exon 19 and in different sites , which are rs1143578253, their polymorphism are TT, TA, and AA and their distribution ratios were 15.38, 56.41 and 28.21%, and that the non-significant difference, and the frequency of the allele was 0.44 and 0.56 for the two alleles C and T, respectively, and the heterogeneity rs1141508235. It appeared that there were two genotypes of the studied piece in the ACTN3/ EXON19 gene in the thoroughbred horses, CC and CT genotype, with the absence of the TT structure, and the highly significant differences (P≤0.01) between the distribution ratios of the combinations in the British thoroughbred, as the two ratios were 87.18 and 12.82% for the two genotypes CC and TC, and the allelic frequencies for the C and T alleles were 0.94 and 0.06, respectively. The distribution percentages of ACTN3/EXON19 gene polymorphism at position rs1146645613/SNP for the horse sample in this study had high significant differences (P≤0.01) between the genotype's distribution percentages in the British thoroughbred, and the percentages were 87.18 and 12.82% for the two genotypes TT and TC, and the mutant structure did not appear. CC and the frequencies for the T and C alleles were 0.94 and 0.06, respectively. There was a significant variation (P≤0.05) in the depth of respiration after exercise according to the different polymorphism of the rs1143578253 hetero, and the highest rate was for the TT combination (84.80 ± 5.13 seconds) as well as the number of respiration times after exercise, but for the hybrid genotype TA (59.16 ± 1.81 times/min), there was There was a highly significant variation (P≤0.01) in the rate of speed according to the genotype of the thoroughbred horses, and the highest rate of speed was for horses with the mutant genotype AA and the lowest for their counterparts with the wild genotype TT. It is concluded from the study that the ACTN3 gene has a distinct role in some of the traits studied on British terrier horses in Iraq, especially the number and depth of respiration after exercise, rate of speed, and some body measurements, specifically body length, chest circumference, and frontal height, as it varied significantly with the difference in the polymorphism of some variations.

Key words: Polymorphism, ACTN3/ EXON19 gene, Thoroughbred horses.

[*] **The research is extracted from the doctoral thesis of the first researcher.**

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المظاهر الوراثية لجين ACTN3 / EXON-19 وارتباطها بأداء خيول الثيربرد في العراق

وليد زين تركي[1]، نصر نوري الأنباري[2]

[1] قسم الإنتاج الحيواني، كلية علوم الهندسة الزراعية، جامعة بغداد، بغداد، العراق، waleed.zaien1101a@coagri.uobaghdad.edu.iq
[2] استاذ ، قسم الإنتاج الحيواني، كلية علوم الهندسة الزراعية، جامعة بغداد، بغداد، العراق، nassr.n@coagri.uobaghdad.edu.iq

الخلاصة

تم اجراء البحث في نادي الفروسية العراقي-Iraqi Equestrian Club (15 كم غرب مركز مدينة بغداد) للمدة من 2021/10/1 ولغاية 2022/11/30، بهدف الكشف عن المظاهر الوراثية في جين ACTN3 منطقة EXON-19 وعلاقتها في اداء خيول الثيربرد البريطانية في العراق. تبين أن هناك 3 تغايرات (3 SNPs) في جين ACTN3 فيما يخص اكسون 19 وفي مواقع مختلفة وهي rs1143578253 مظاهرها TT و TA و AA أذ كانت نسب توزيعها ذات فروق غير معنوية ونسبها 15.38 و 56.41 و 28.21% وبتكرار اليلي بلغ 0.44 و 0.56 للاليلين T و A على التوالي والتغاير rs1141508235 وظهر وجود تركيبين وراثيين من القطعة المدروسة في جين ACTN3/ EXON19 في خيول الثيربرد وهما CC و CT مع غياب التركيب TT وان الفروق عالية المعنوية (P≤0.01) بين نسب توزيع التراكيب في الثيربرد البريطاني، إذ كانت النسبتين 87.18 و 12.82 % للتركيبين الوراثيين CC و TC وبلغ التكرار الاليلي للاليلين C وT 0.94 و 0.06 على التوالي. كانت نسب توزيع التراكيب الوراثية لجين *ACTN3*/EXON19 الموقع الوراثي rs1146645613/SNP لعينة الخيول في هذه الدراسة ذات فروق عالية المعنوية (P≤0.01) بين نسب توزيع التراكيب في الثيربرد البريطاني، وكانت النسبتين 87.18 و 12.82 % للتركيبين الوراثيين TT و TC وعدم ظهور التركيب الطافر CC وبلغ التكرار للاليلين T وC 0.94 و 0.06 على التوالي. كان هنالك تباينا معنويا (P≤0.05) في عمق التنفس بعد التمارين باختلاف المظاهر للتغاير rs1143578253 وكان اعلى معدل للتركيب TT (84.80 ±5.13 ثانية) وكذلك عدد مرات التنفس بعد التمارين لكن للتركيب الوراثي الهجين TA (59.16 ±1.81 مرة/ دقيقة)، هنالك تباينا عالي المعنوية (P≤0.01) في معدل السرعة بأختلاف التركيب الوراثي لخيول الثيربرد، وبلغ اعلى معدل للسرعة للخيول ذات التركيب الوراثي الطافر AA وادناها لمثيلاتها ذات التركيب الوراثي البري TT . يستنتج من الدراسة إن جين ACTN3 له دورا مميزا في بعض الصفات المدروسة على خيول الثيربرد البريطاني في العراق لاسيما عدد وعمق التنفس بعد التمارين ومعدل السرعة وبعض قياسات الجسم وتحديدا طول الجسم ومحيط الصدر والارتفاع من الامام، إذ تباينت معنويا باختلاف المظاهر الوراثية لبعض التغايرات.

الكلمات المفتاحية: المظاهر الوراثية المتعددة، جين ACTN3/EXON19 ، خيول الثيربرد.

INTRODUCTION

Purebred racing horses (Thoroughbred horses) are warm-blooded domesticated horses that are frequently used in racing, as they are characterized by their agility and speed over many other horse breeds, including local ones **(Bower, *et al.*, 2011)**. In the seventeenth and eighteenth centuries, the royal family in Britain adopted the thoroughbred horses in the races, and then spread to Europe, the Americas, Asia and Australia **(Rooney, 2017)**. Various criteria have been used to determine the optimal speed in racing horses by relying on accurate speed data obtained from different races **(Rieder, *et al.*, 2010; Athmar, *et al.*, 2015 ; AL-Zaiadi & AL-Shekdhaher, 2016; Towfik, *et al.*, 2017 ; Turki & Al-Anbari, 2018; Salam *et al.*, 2022)**, and developing a mathematical model that provides information on how horses regulate their speed and effort over a given distance **(Mercier & Aftalion, 2020)**, Endurance performance was also measured, as horses can achieve an average speed in endurance races exceeding 25 km / h, especially in the last stage of the race **(Adamu, *et al.*, 2012; Nagy, *et al.*, 2012;Gurgul, *et al.*, 2019)** reported that the identification of improvement programs in horses is most likely associated with selection Which focuses on improving horse riding and racing based on genetic variants obtained using Illumina microarray technology, also known as Bead Array or Bead Chip technology, in horses. The sarcomere α-actinin proteins, encoded by the ACTN2 and ACTN3 genes, are major structural components of Z-line proteins and have high sequence

similarity: α-actinin2 is found in all skeletal muscle fibers, while α-actinin3 has evolved as a result of specialized expression in type 2 fibers. The second only which is fast glycogenolysis **(Thomas, *et al.*, 2014)**. Functional and structural analysis of the ACTN3 gene has been conducted in relation to its association with horse performance characteristics, and so far many researches have focused on identifying SNP differences in the ACTN3 gene for horses in different breeds **(Thomas, *et al*., 2014 and Wang, 2018).**The study of genetic variation within and among individuals enables researchers to obtain important information about any organism, which may not be available in the light of traditional methods, as well as help in finding and describing levels of genetic variation, and then obtaining basic information about the composition of the population **(Hamdallah, 2009 ; Ahmed, *et al*., 2018; Hamed & Abbas, 2020; Hadi, *et al*., 2020 ; Khalaf, *et al*., 2022).** The research aims to determine the genetic polymorphism of the actin gene in a sample of British terrier horses in Iraq and to detect variations in the ACTN3 / EXON-19 gene, and the relationship of ACTN3 gene polymorphism in some physiological characteristics, body dimensions, rate of speed, and the concentration of the actin gene.

MATERIALS AND METHODS:

The study was conducted in the Iraqi Equestrian Club located in Baghdad / Al-Amriya Governorate, on a sample of British terriers in the races that take place in the club, from 1/2/2022 to 30/12/2022, with the aim of extracting DNA and detecting ACTN3 gene polymorphism /region. EXON-19 and its relation to the performance of terrier horses in Iraq.

Collecting blood samples:

10 ml of blood was collected from the jugular vein of each animal, and the blood was divided into three tubes. The first tube was added with an EDTA anticoagulant produced by the Jordanian AFCO (Al-Hanoof Factory), and transferred in a refrigerated container to the laboratory for safekeeping. By freezing at - 4 °C until the time of DNA extraction, omeprazole was added to it to benefit from it in knowing gene expression, and the latter was waited 15 minutes until the state of clotting occurred, after which a centrifugation was performed at a speed of 3000 rpm for 5 minutes, and the serum was separated from hemoglobin and transferred The serum is taken with the first tube in a refrigerated box to the laboratory to conduct the necessary analysis to find out the required blood characteristics.

Gene Molecular Analysis:

For the purpose of conducting the molecular analysis of the studied gene on the blood samples drawn from the studied horses that were preserved by freezing, they were taken out of the freezer for the purpose of conducting laboratory analyzes to know the genotypes of the genes. According to Temp.C=63 and Product by 870 bp. Suitable primer designed in laboratory.

F:GCAGATGCAGAGATGTGAT
R: TCCTCCTCCTGTTCCATATAC

DNA Extraction:

Genomic DNA was isolated from the blood sample according to the Relia Prep™ Blood gDNA Miniprep System, PR omega protocol. A quantitative fluorometer was used to detect the concentration of extracted DNA in order to screen for sample quality for downstream applications. For 1 µl of DNA, 200 µl of diluted Quantifluor dye was mixed. After 5 minutes of incubation at room temperature, DNA concentration values were detected.

PCR program:

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	63	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

After PCR amplification, agarose gel electrophoresis was performed to confirm the presence of amplification. The polymerase chain reaction (PCR) was completely based on the standards of the extracted DNA.

1 X TAE buffer ،DNA ladder marker ،Ethidium bromide (10mg / ml).

Sequence analysis technology was performed to determine genotypes and detect the presence of mutations by sending samples to South Korea, by sending PCR products for Sanger sequencing using the ABI3730XL device, to know the sequences of automated DNA nitrogenous bases, by Macrogen Corporation - in Korea, and the results were received were emailed and then analyzed using genetic software.

Statistical analysis:

The data were analyzed statistically using the program Statistical Analysis System-**SAS (2018)** to study the effect of the genetic polymorphism of the actin gene (ACTN3) of the EXON-19 region on the studied traits, and the significant differences between the averages were compared using Duncan's multinomial test by applying the least squares means procedur, and the Chi-square- $\chi 2$ test was used to compare the distribution percentages of genotypes for each SNP in the ACTN3/EXON-19 gene.

The statistical model was as follows/ SNP's of ACTN3-EXON-19 gene:

A: rs1143578253 /SNP1

$Y_{ij} = \mu + G_i + e_{ij}$

Where:

Yij = dependent variable.

μ = overall mean.

G_i= Effect of Genotype (TT, TA and AA).

e_{ij} = Error term.

The statistical model above has been applied on rs1141508235 /SNP2 (CC, and CT genotype) and rs1146645613 /SNP3 (TT and CC genotype).

RESULTS AND DISCUSSION:

The DNA was extracted and the studied piece of the ACTN3 / EXON19 gene was duplicated within the sequencing technology with the loading dye of agarose gel (3%) with voltage, current and time adjustments, and then photographed the result to ensure the success of the doubling process and obtain the required piece, which was the size of 870 pairs basally (Fig. 1).

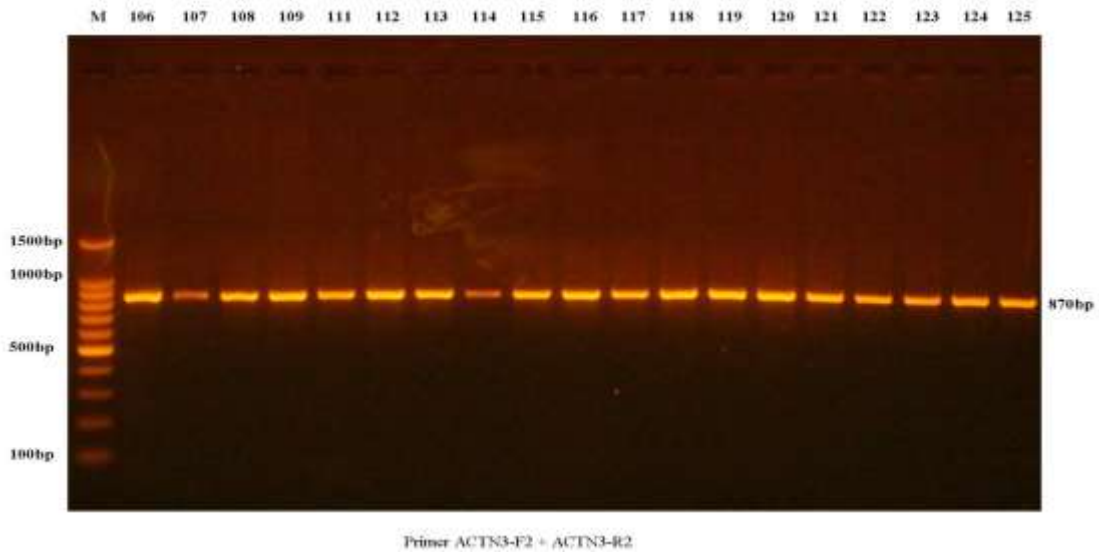

Figure (1): Results of amplification of the exon 19 (F2) region of ACTN3 gene/ Equus caballus for samples (106-125). The PCR result was that the sample size was (870 bp).

Genotype and allele frequency:

rs1148960207 // ACTN3: EXON19:

It is clear from (Table, 1) and (Figure, 2) the distribution ratios of the ACTN3/EXON19 gene polymorphism at locus rs1148960207/SNP1 for the horse sample in this study, as the ratios were 15.38, 56.41, and 28.21%, with an allele frequency of 0.44 and 0.56 for each of the T and A alleles. Respectively, the analysis of rs1143578253 SNP of ACTN3 gene/exon 19 using Sanger sequencing is illustrated in Figure 2. The results of the distribution of genotypes and allelic frequency differ according to the regions of the gene, the size of the segment, the study site, the number or size of the sample included in the study, as well as the role of chance **(Mukund & Subramaniam , 2020 ; Al-Sarai & Al-Anbari, 2021).**

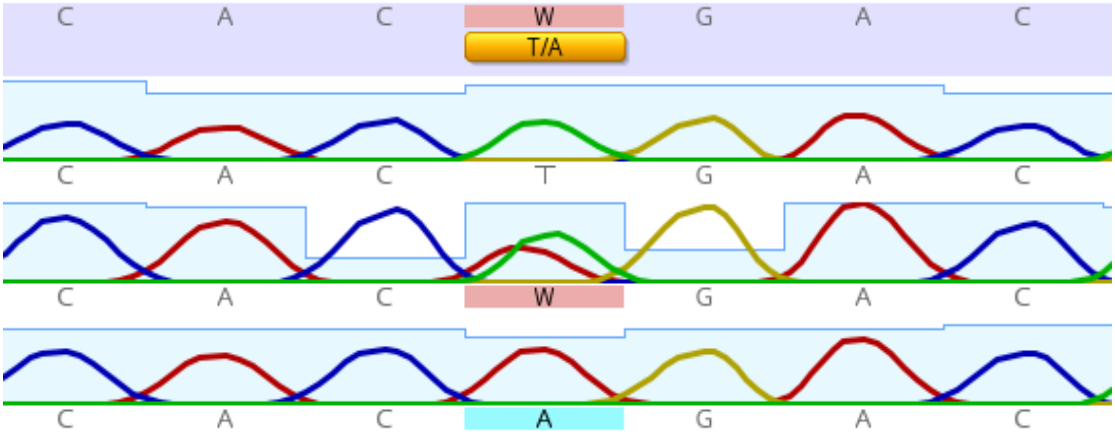

Figure (2): Analysis of the rs1143578253 SNP of the ACTN3 gene/exon 19 using Sanger sequencing. The "T" allele and its quality with the A allele indicates that the gene is homozygous. The presence of the "T" allele with the T alleles indicates the homozygous gene, and the presence of "T" and "A" indicates that the combination is T/A heterozygous.

rs1141508235 // ACTN3: EXON19:

It is clear from Table (1) and Figure (3) that there are two genotypes from the studied piece in the titration rs1141508235 SNP of the ACTN3/ EXON19 gene in the thoroughbred horses, namely CC and CT, with the absence of the TT structure, and that the differences are highly significant (P≤0.01)) between the ratios of the distribution of the combinations In the British thoroughbred, the percentages were 87.18 and 12.82% for the two genotypes CC and TC, and the TT mutation did not appear, and the allelic frequency of the C and T alleles was 0.94 and 0.06, respectively.

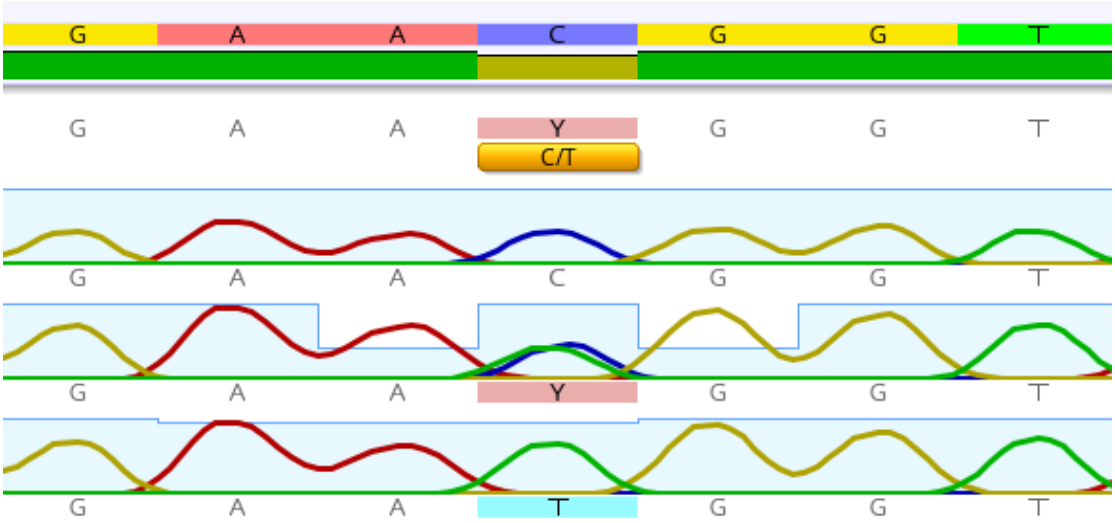

Figure (3): Analysis of the rs1141508235 SNP of the ACTN3 gene/exon 19 using Sanger sequencing. The "C" allele and its quality with the C allele indicates that the gene is homozygous. The presence of the “T” allele with the T alleles indicates the homozygous gene, and the presence of “C” and “T” indicates that the combination is C/T heterozygous.

rs1146645613 // ACTN3: EXON19

It is clear from (Table, 1) the distribution ratios of ACTN3/EXON19 gene polymorphism at position rs1146645613/SNP for the horse sample in this study. The highly significant differences were (P≤0.01) between the distribution ratios of genotypes in the British thoroughbred, and the percentages were 87.18 and 12.82% for the two combinations. The TT and TC genes did not appear, and the CC mutated structure did not appear. The frequency of the T and C alleles was 0.94 and 0.06, respectively. Figure 4 show the analysis of rs1146645613 SNP of the ACTN3 gene/exon 19 using Sanger sequencing.

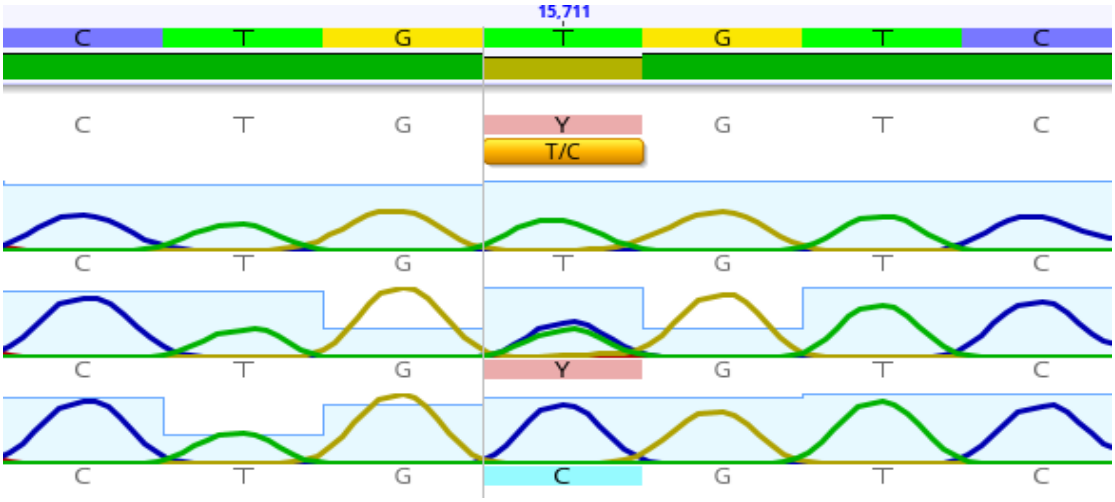

Figure (4): Analysis of the rs1146645613 SNP of the ACTN3 gene/exon 19 using Sanger sequencing. The "C" allele and its quality with the C allele indicates that the gene is homozygous. The presence of the "T" allele with the T alleles indicates the homozygous gene, and the presence of "C" and "T" indicates that the combination is C/T heterozygous.

Table (1): Genotype distribution and allele frequency in ACTN3-EXON-19 gene in Local horses

SNPs	Genotype	No	(%)	Allele	Frequency
rs1143578253 /SNP1	TT	6	15.38	T	0.44
	TA	11	56.41		
	AA	22	28.21	A	0.56
	Total	39	100%		
	χ^2	--	1.923 NS	--	--
rs1141508235 /SNP2	CC	34	87.18	C	0.94
	CT	5	12.82		
	TT	0	0.00	T	0.06
	Total	39	100%		
	χ^2	--	8.052 **	--	--
rs1146645613 /SNP3	TT	34	87.18	T	0.94
	TC	5	12.82		
	CC	0	0.00	C	0.06
	Total	39	100%		
	χ^2	--	8.052 **	--	--
** (P≤0.01), NS: Non-Significant.					

Relationship of ACTN3/EXN19 gene polymorphism with parameters rs1143578253/ SNP1// ACTN3: EXON19:

It is noted from (Table, 2) the relationship of ACTN3 gene polymorphism /EXON19 rs1143578253 with the studied traits on the British terrier horses, as there was a significant variation (P≤0.05) in the depth of breathing after exercise, and the highest rate of TT was (84.80 ± 5.13 seconds) and so on. The number of respirations after exercise but for the TA hybrid genotype (59.16 ± 1.81 times/min). There is a highly significant variation (P≤0.01) in the rate of speed according to the genotype of the thoroughbred horses. The highest rate of speed was for horses with the mutant genotype AA (2361.46 ± 1236.06 m/min) and the lowest for those with the wild genotype AA (1105.08 ± 24.65 m/min). While the rest of the traits were not significantly affected by ACTN3-EXON19 polymorphism// rs1143578253. **Dominguez & Holmes (2011)** indicated that actin is the most abundant protein in most eukaryotic cells, as it is highly available and participates in more protein-to-protein interactions than any known protein, these properties along with its ability to transition between the globular state (G-actin) F-actin (filamentous), which is under the control of hydrolysis of nucleotides, ions, and a large number of actin-binding proteins, makes actin an important role in many cellular functions, ranging from cell motility.

Table (2): Relationship of ACTN3-EXON-19 gene polymorphism/ rs1143578253 SNP1 and parameters study in Thoroughbred

Parameters	Mean ± SE			Level of Sig.
	TT	TA	AA	
Depth of respiration before exercise (sc)	23.40 ±4.46	20.81 ±0.60	21.54 ±1.24	N.S.
No of respiration before exercise (min.)	12.60 ±0.74	14.48 ±0.39	14.00 ±0.52	N.S.
Depth of respiration after exercise (sc)	84.80 ±5.13 a	72.28 ±2.41 b	73.10 ±3.43 b	*
No of respiration after exercise (min.)	50.40 ±3.20 b	59.16 ±1.81 a	57.72 ±3.36 ab	*
Rate of speed (m/min.)	1105.08 ±24.65 b	1616.79 ±557.86 ab	2361.46 ±1236.06 a	*
Body length (cm)	143.60 ±2.40	147.51 ±2.87	151.23 ±4.14	N.S.
Heart girth (cm)	149.25 ±1.25	155.73 ±4.29	156.21 ±5.12	N.S.
Height from the front (cm)	151.30 ±1.20	154.56 ±2.78	158.91 ±3.61	N.S.
α-ACTN3 conc. (ng/ml)	12.36 ±0.91	11.82 ±1.27	11.32 ±0.94	N.S.
Means having with the different letters in same row differed significantly. * (P≤0.05).				

rs1141508235/ SNP2 // ACTN3: EXON19:

It is noted from (Table, 3) the relationship of ACTN3 gene polymorphism /EXON19 rs1141508235 with the studied traits on the thoroughbred horses, and the variation was not significant with the difference of the genotype except for the height at the front (P≤0.05), as the maximum rate for horses with the hybrid genotype was CT (166.40 ± 4.60 cm), while the rate was 152.97 ± 2.11 cm for their wild CC counterparts. This may be due to the variation in muscle contraction and stretching and the contractile sarcomere movement, so that actin has a role in many cellular functions, which may be reflected in some dimensions of the body, including the height from the front, depending on the genetic makeup **(Mukund & Subramaniam, 2020).**

rs1146645613/ SNP3 //ACTN3: EXON19:

It is noted from (Table, 4) the relationship of ACTN3 gene polymorphism /EXON19 rs1146645613 with the studied traits on thebred horses, and there was a significant variation (P≤0.05) in the rate of speed according to the genotype, as the highest rate of speed was for horses with the wild genotype TT (1756.26 ± 369.18 m / min), while the rate was 1130.70 ± 4.18 m / min for those of the hybrid genotype TC, and the difference in the speed rate according to ACTN3-EXON19 rs1148960207/ gene for purebred horses can be attributed to the variation in the expression of α-actinin-3 in fast glycolytic muscle fibers, which are necessary for rapid muscle contraction. This result indicates the possibility of improving the rate of speed in horses through selection for individuals carrying the C allele or the CT genotype, and it is possible to consider the ACTN3 gene. One of the best candidate genes as a genetic marker for improving the speed of horses is that it has a major effect on this trait for most of the variants obtained **(Mukund & Subramaniam, 2020).** While the rest of the studied traits were not significantly affected by the different genotypes of the ACTN3-EXON19 gene rs1146645613/ of the thoroughbred horses.

Table (4): Relationship of ACTN3-EXON-19 gene polymorphism/ rs1146645613 SNP3 and parameters study in Thoroughbred

Parameters	Mean ± SE		Level of Sig.
	TT	TC	
Depth of respiration before exercise (sc)	21.34 ±0.79	21.16 ±1.42	N.S.
No of respiration before exercise (min.)	14.08 ±0.34	14.33 ±0.42	N.S.
Depth of respiration after exercise (sc)	73.31 ±2.19	78.01 ±2.25	N.S.
No of respiration after exercise (min.)	57.97 ±1.65	56.17 ±4.01	N.S.
Rate of speed (m/min.)	1756.26 ±369.18 a	1130.70 ±4.18 b	*
Body length (cm)	148.03 ±2.18	152.95 ±13.05	N.S.
Heart girth (cm)	155.09 ±3.24	154.85 ±6.75	N.S.
Height from the front (cm)	154.97 ±2.11	164.40 ±4.63	N.S.
α-ACTN3 conc. (ng/ml)	10.95 ±0.84	11.67 ±0.97	N.S.
Means having with the different letters in same row differed significantly. * (P≤0.05).			

CONCLUSION

From the study that the ACTN3 gene has a distinct role in some of the traits studied on British terrier horses in Iraq, especially the number and depth of respiration after exercise, rate of speed, and some body measurements, specifically body length, chest circumference, and frontal height, as it varied significantly with the difference in the polymorphism of some variations.

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