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Article

Molecular and morphological characterization of the seed gall nematodes *Anguina tritici* from central and southern Iraq

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ABSTRACT

This is the first morphological investigation of the wheat gall nematodes *Anguina tritici* found on wheat in southern and midland areas of Iraq. A survey was conducted, and samples were collected from wheat fields in eight governorates (Diyala, Baghdad, Qadisiyah, Najaf, Wasit, Maysan, Dhi Qar and Basra, 3 regions each and three fields for each region during the 2020-2021 growing season. Wheat-gall nematodes were extracted and observed under a stereomicroscope. Nematodes of interest were hand-picked, identified and photo-micro-graphed using a microscope equipped with a digital camera. Two species of anguinid nematodes (Anguinidae: Tylenchida) were found and characterized. This species is characterized by its 810-905 mm long body, body slightly curved to almost straight, with a 9-10 µm small stylet with delicate lip region very slightly offset, pharyngeal lobe long, wider than adjacent body part and 16-17µm broad, distance of dorsal gland orifice to the style 2-4, position of dorsal gland orifice to the style base expressed as a percentage of the total style length20-40 µm. Anterior end to the valve of the median bulb 67 -72µm; anterior end to excretory. Tail long, gradually tapering to an acute tip without mucro 61 -70 µm. As for the molecular identification and comparison with the GenBank, sequencing of Dhi Qar samples showed having three transitions at sites 59,125,168 G\T, G\T, and G\A, respectively, and one transversion at 140 G\C site. It was found that the ITS gene has a compatibility of 98% with the original sequence conserved in the Genbank with ID KC818619. As for the *Anguina tritici* isolated from Basra, it contains two transitions at locations 247 and 253 C\T, four transversions 238,245,249, 251 G\T, G\C, A\C, and T\A, respectively, with similarity of 97%. Maysan samples were compatible with 98% and had one transition at 168 G\A site and three transversions at the locations 125,128.25 G\T. ITS gene from Diyala showed 98% compatibility with the sequence in GenBank, having one transition at locus G\A 93 and three transversions at 83,78,59 G\T, G\C, and G\T, respectively. Two transversions in locations 59,117 nucleotide G\T, A\C in Al-Kut samples, and at 125,142 G\T, G\C in Baghdad ones, while Najaf samples had one transversion, and one transition in sites 213, 234 G\C, C\T respectively, as all of them showed 99% similarity with the original sequence in GenBank.

Keywords: Field crop, PCR, PPN, wheat**.**

INTRODUCTION

Anguina tritici was discovered in England in 1743 and was the first plant parasitic nematode to be recognized. [1]. Where it was primarily found in wheat but also rye to a lesser extent. [2]. It is one of the major aerial diseases. It causes sustainable losses in the wheat crop of tropical and sub-tropical countries. [3]. It can be found anywhere wheat is grown. This disease is still common in Eastern Europe, and in parts of Africa and Asia, symptoms of nematode attack can be discerned at the seedling stage. However, farmers generally fail to recognize the disease before harvesting and threshing of the plant [4].

A.tritici has remained an important nematode pest in Iraq, occurring in most wheat-growing areas, causing a reduction in the wheat yield, which increased to 75% on the same cultivar in Duhok Province in 1989 [5]. As reported by [3], the percentage of infestation by galls reached its maximum value (50%) in bread wheat in Bashika, northern Iraq. His disease reduces the human consumption and market price of wheat (Madhi *et al.*,2021)[6]. During the survey in Duhok Province in 2010–2011, it was shown that *A. tritici* was still one of the major problems in this region, reaching 50% in some wheat fields [7]. Approved Method for Pest Surveillance: Initial diagnosis will be via morphology. All initial diagnoses will need to be confirmed with molecular methods. A dichotomous key has been developed to identify Anguina tritici [8] that uses many morphological and biological characteristics. Although not officially validated for regulatory purposes, [9] demonstrated PCR primer pairs that target specific areas of the ITS2 region and can differentiate *A. tritici from A. agrostis, A. funesta, and A. pacificae*. The PCR assay can be used for single nematodes. The detection of other undescribed Anguina species should also be possible by the Anguina genus-specific primers included in this assay. This assay can be used for screening Anguina tritici morphological suspect samples[9].

MATERIALS AND METHODS

Various Nematode samples

A survey of wheat fields was conducted in eight governorates during the 2020-2021 agricultural season. The surveyed governorates represent about 43% of the total area and production of wheat in Iraq (ICSO, 2020)[10].

Seventy-two fields were surveyed distributed over the eight governorates as shown on the map: Diyala, Baghdad, Qadisiyah, Najaf, Wasit, Maysan, Dhi Qar and Basra, with 3 regions and each region has three fields, as shown in figure (1). At least twenty sub-samples were collected to generate a composite pool sample. Infected plants were taken. Sampling sites were chosen randomly, avoiding areas that showed pressure by equipment (e.g., tractors, combines, trucks, etc.).

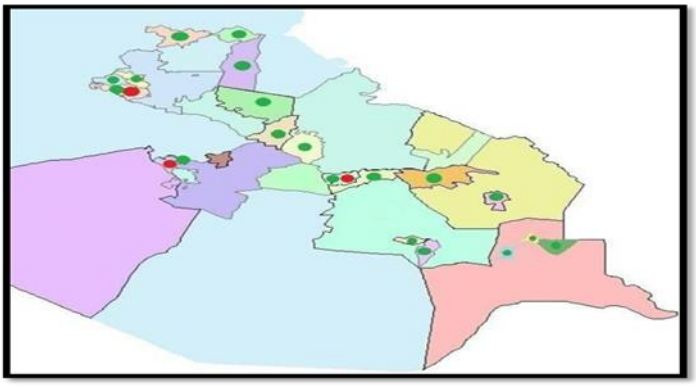

Fig 1: Distribution of Nematode samples over the eight governorates.{1}

Morphological Identification

The extracted nematodes were observed under a stereomicroscope, and nematodes of interest were hand-picked, then killed, fixed and transferred to anhydrous glycerin, the method proposed by De Grisse (1969)[11]. Permanent slides and morphometric data were obtained using a drawing tube attached to an Olympus BH-2 light microscope. Photomicrographs were made using the same microscope equipped with a digital camera (Golhasan *et al.*, 2016)**[12]**. Morphological and morphometric investigation of every known species of *A. tritici* is based on a thorough review and collection of the literature on the subject.

DNA Extraction

Genomic DNA from nematodes was extracted using a (DNA micro kit given by G-spin DNA extraction kit, Korea) according to the manufacturer's instructions; primers were obtained from the IDT corporation for this study.

The Amplification of Target Nucleic Acid DNA

By using the primers F (5'- TCCGTAGGTGAACCTGCGG-3') and R (5'-TCCTCCGCTTATTGATATGC-3') (Mohammed et al.,2021)[13], the ITS gene was amplified. The PCR reaction mixture comprises 5 µl of pre-Master Mix, 1.5 µl DNA, 1 µl of each forward and reverse primer, and the volume is completed to 25 µl by deionized water. The thermal cycling conditions are shown in Table 2. A 70 volts/65 amps current is applied to the gel for 60 minutes. Following electrophoresis, a UV trans illuminator was used to see the results. The gene sequencing was carried out at Microgen company using their ABI 3730xl genetic analyzer. The Basic Local Alignment Search Tool (BLAST) tool conducted a homology search online at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and Bio Edit.

Phase	Tm (°C)	Time	No. of cycle
Initial Denaturation	**94°C**	**3 min.**	**1 cycle**
Denaturation -2	**94°C**	**45sec**	**35 cycle**
Annealing	**52°C**	**1 min**	
Extension-1	**72°C**	**1 min**	
Extension -2	**72°C**	**7 min.**	**1 cycle**

E. **Sequencing and Sequence Alignment:**

After staining with Red Stain, the PCR products were separated on a 2 percent agarose gel electrophoresis and observed by exposure to ultraviolet light (302 nm) **(Aswad et al.,2021)[14]**. Macrogen Korea performed the conventional PCR to sequence the Internal Transcribed Spacer (ITS) gene, using 25µl of the reaction volume and 50µl of primers. A homology search was then carried out using the Basic Local Alignment Search Tool (BLAST) program, which is available online at the National Center for Biotechnology Information (NCBI) at (http://www.ncbi.nlm.nih.gov) and Bio Edit program.

PCR products were isolated and sequenced forward using an ABI 3130 Genetic Analyzer (Applied BioSystems, Foster City, CA) and the Big Dye Terminator v3.1 Cycle Sequencing kit.

The sequence was examined in the nucleotide databases using the NCBI's Basic Local Alignment Search Tool Bio ID software (ID) to identify the sample and submit it to Gene Bank. The Bio ID software acquired related sequences from the sample from the NCBI's nucleotide database (www.ncbi.nlm.gov/nucleotide).

RESULTS

Morphometrics

The holotype and paratypes second stage juveniles This species is characterized by its 810-905 mm long body. The body is slightly curved to almost straight, Stylet 9-10 µm small with delicate Lip region very slightly offset, Pharyngeal lobe long, wider than adjacent body part and 16-17µm broad, a distance of dorsal gland orifice to the style 2-4, the position of dorsal gland orifice to the style base expressed as a percentage of the total style length20-40 µm. Anterior end to the valve of the median bulb 67 -72µm; anterior end to excretory. Tail long, gradually tapering to an acute tip without mucro 61 -70 µm (Table 2).

	Female	
Characters	Holotype	Paratype
N	-	10.0
L	870 809.0	866.2 ± 29.2 (810-905) 801.0 ± 28.1 (744-839)
L'	54.4	53.5 ± 2.1 (49.6-56.6)
A	4.7	4.5 ± 0.1 (4.3-4.7)
B	4.5	4.3 ± 0.1 (4.1-4.5)
b'	14.3	13.5 ± 0.6 (12.3-14.3)
+	4.4	5.1 ± 0.4 (4.4-5.6)
c'	2.0	2.0 ± 0.0 (2.0-2.0)
Head Height	5.0	4.9 ± 0.3 (4.0-5.0
Head Width	9.0	9.8 ± 0.4 (9.0-10.0)
Stylet	3.0	3.9 ± 0.3 (3.0-4.0)
Conus	33.3	39.8 ± 2.7 (33.3-44.4)
M	3.0	3.1 ± 0.6 (2.0-4.0)
DGO	33.3	31.7 ± 5.7 (20.0-40.0)
O	67.0	70.5± 1.4(67.0-72.0)
Median bulb	36.2	36.6 ± 0.9 (35.0-38.0)
MB	124.0	130.3 ± 5.8(123-144)
Excretory pore	185.0	192.7 ± 4.9 (185-200)
Oesophagus.	193.0	199.4 ± 5.0 (193-208)
End Of Glands	16.0	16.2 ± 0.4 (16.0-17.0)
Body Width(BW)	14.0	12.7 ± 0.7 (12.0-14.0)
Anal Body Width	61.0	64.4 ± 2.9 (61.0-70.0)

Table 2: Morphometric second-stage juveniles of *A. tritici* sampled from southern and mid-land areas of Iraq All measurements in µm and the form: Mean ± SD (range). The holotype and paratypes were glycerin-mounted on permanent slides *Abbreviations: a = body length/greatest body diameter; b = body length/distance from anterior end to pharynx-intestinal junction; c = body length/tail length; c′ = tail length/tail diameter at anus or cloaca; L = overall body length; n = number of specimens on which measurements are based; DGO=distance of dorsal gland orifice to the style; O= position of dorsal gland orifice to the style base expressed as a percentage of the total style length.

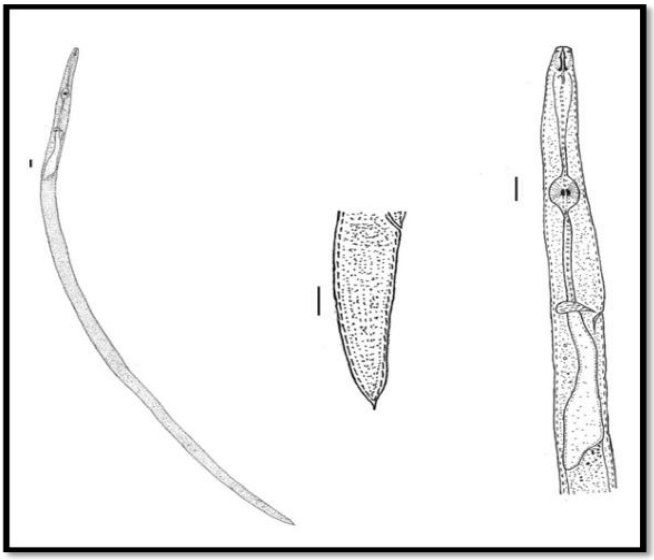

Figure 2. Anguina tritici. n. second-stage juveniles. A: second stage juveniles entire; B: Head and stylet; C, Tail tips;. (Scale bars: A B C = 10µm.

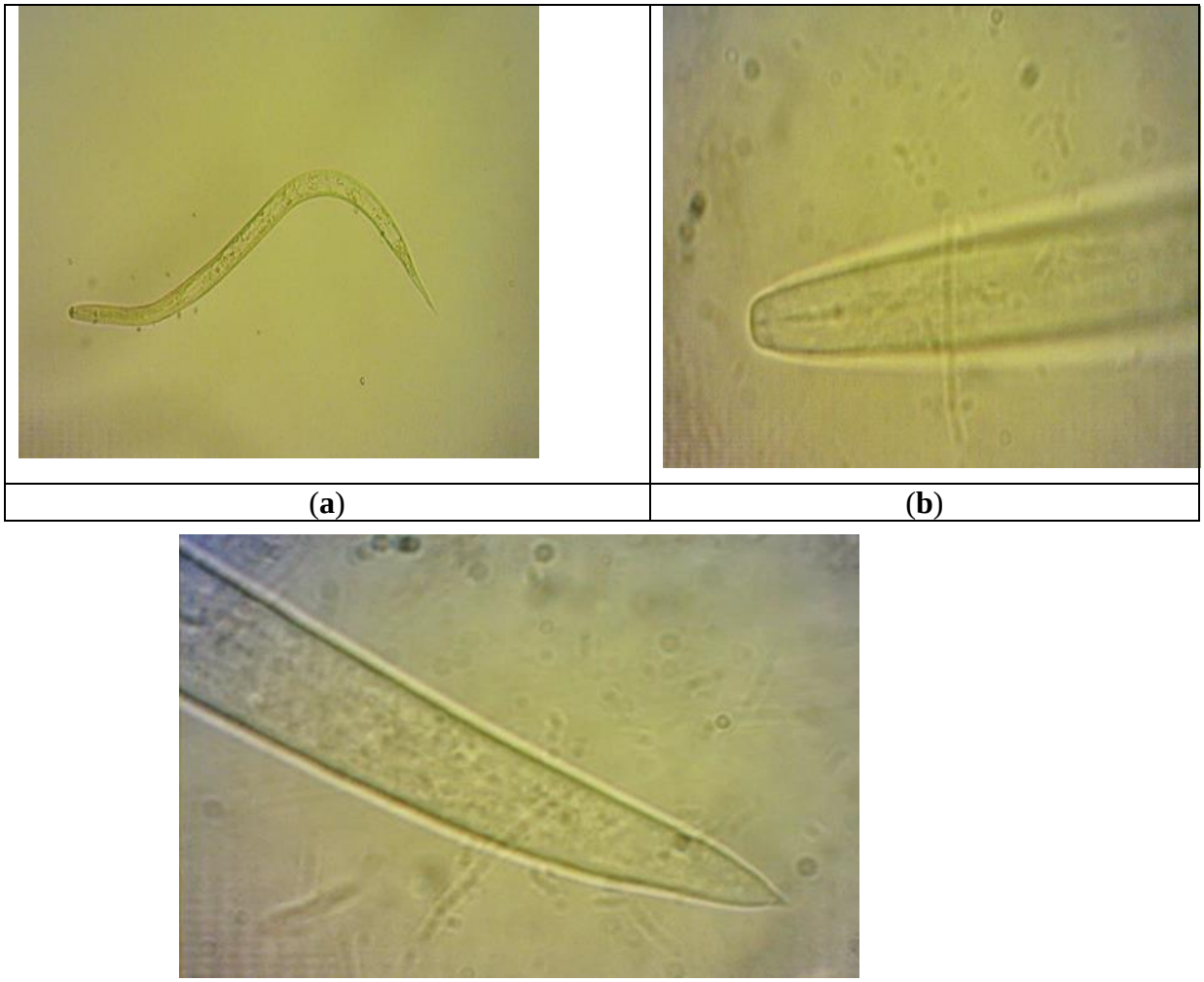

Figure. 3. *Anguina tritici* (A) J2 entire body; (B) anterior part; (C) body portion posterior to the vulva. Scale bars ¼ 20mm

Molecular identification

The polymerase chain reaction diagnostic techniques are rapid and easy; because of their high sensitivity and specificity methods for detecting particular sequences of nucleic acids, they are quickly becoming the most widely used of all molecular genetics methods for identifying the Nematode Species, PCR products of 650bp for *Internal Transcribed Spacer (ITS)* gene as seen in figure (3).

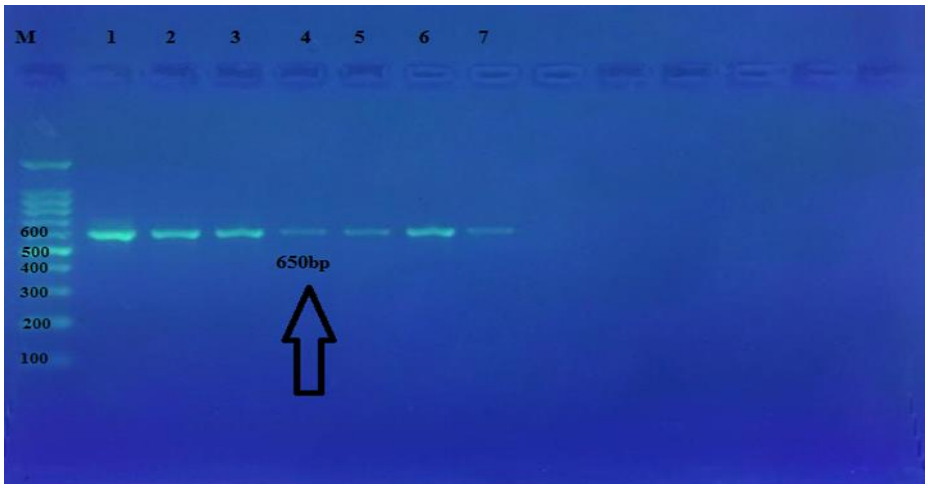

Figure (3):- Agarose gel electrophoresis for *ITS* gene (650bp). Bands were fractionated by electrophoresis on a 2% agarose gel (2 h.,1X TBE) and visualized under UV light after staining with red stain. Lane: M (M: 100bp ladder).

The *ITS* gene was amplified by PCR and sent to Macrogen Company Korea for sequencing service. The sequence was deposited in the GenBank database under accession numbers **(https://www.ncbi.nlm.nih.gov/nuccore/ /MW331328.1/MW331327.1/MW331326.1/MW331323.1/MW331324.1/ MW331322.1/ MW331325.1).**

The results of the *Anguina tritici* nitrogenous base sequence that was isolated from the Dhi Qar governorate showed that it contains three Transition at sites 59,125,168 G\T, G\T, and G\A respectively, and one Transvertion at the 140 G\C site after comparing it with the GenBank. It was found that the *ITS* gene has a compatibility of 98% with the original sequence conserved in the Genbank with ID KC818619.

As for the *Anguina tritici* was isolated from Basra Governorate, it contains two transitions at locations 247 and 253 C\T and four of the Transvertion 238,245,249, 251 G\T, G\C, A\C, and T\A, respectively, which has a similarity of 97%. Maysan Governorate, was found compatible 98% and having one transition at the 168 G\A site and three Transvertion at the locations, 125,128.25 G\T, while the analysis of the results from Diyala governorate showed 98% of compatibility with the sequence in GenBank having one Transition at locus G\A 93 and three Transvertion at the sites, 83,78,59 G\T, G\C, and G\T, respectively, while Al-Kut governorate showed two Transvertion in locations 59,117 nucleotide G\T, A\C, also Baghdad governorate two Transvertion in sites 125,142 G\T, G\C, but Najaf governorate one Transvertion, and one Transition in sites 213,234 G\C, C\T respectively, all of them showed 99% similarity with the original sequence in GenBank, as shown in table (4).

	Type of substitu-tion	Location	Nucleotide	Sequence ID with compare	Sequence ID with submission	Identities
Dhi Qar	**Transition**	**59**	**G\T**	**KC818619.1**	**MW331327.1**	98%
	Transition	**125**	**G\T**			
	Transver-tion	**140**	**G\C**			
	Transition	**168**	**G\A**			
Basra	**Transver-tion**	**238**	**G\T**	**KC818619.1**	**MW331328.1**	97%
	Transver-tion	**245**	**G\C**			
	Transition	**247**	**C\T**			
	Transver-tion	**249**	**A\C**			
	Transver-tion	**251**	**T\A**			
	Transition	**253**	**C\T**			
Maysan	**Transver-tion**	**59**	**G\T**	**KC818619.1**	**MW331326.1**	98%
	Transver-tion	**125**	**G\T**			
	Transver-tion	**128**	**G\T**			
	Transition	**168**	**G\A**			
Diyala	**Transver-tion**	**59**	**G\T**	**KC818619.1**	**MW331323.1**	98%
	Transver-tion	**78**	**G\C**			
	Transver-tion	**83**	**G\T**			
	Transition	**93**	**G\A**			
Kut	**Transver-tion**	**59**	**G\T**	**KC818619.1**	**MW331324.1**	99%
	Transver-tion	**117**	**A\C**			
Baghdad	**Transver-tion**	**125**	**G\T**	**KC818619.1**	**MW331322.1**	99%
	Transver-tion	**142**	**G\C**			
Najaf	**Transver-tion**	**213**	**G\C**	**KC818619.1**	**MW331325.1**	99%
	Transition	234	C\T			
		Location	Nucleotide			

Table (4): Represent type of polymorphism of ITS gene.

The phylogenetic tree was diagrammatic by Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0. As for the Anguina tritici nematode samples studied in the governorates of Iraq (Dhi Qar, Basra, Maysan, Diyala, Al-Kut,

Baghdad, and Najaf), the genetic dimension was 0.9763, which is close to the genetic dimension of the samples in Malaysia and known in the database by the number (ID: KT900693.1) was equal to 1.0076. As shown in Figure (4).

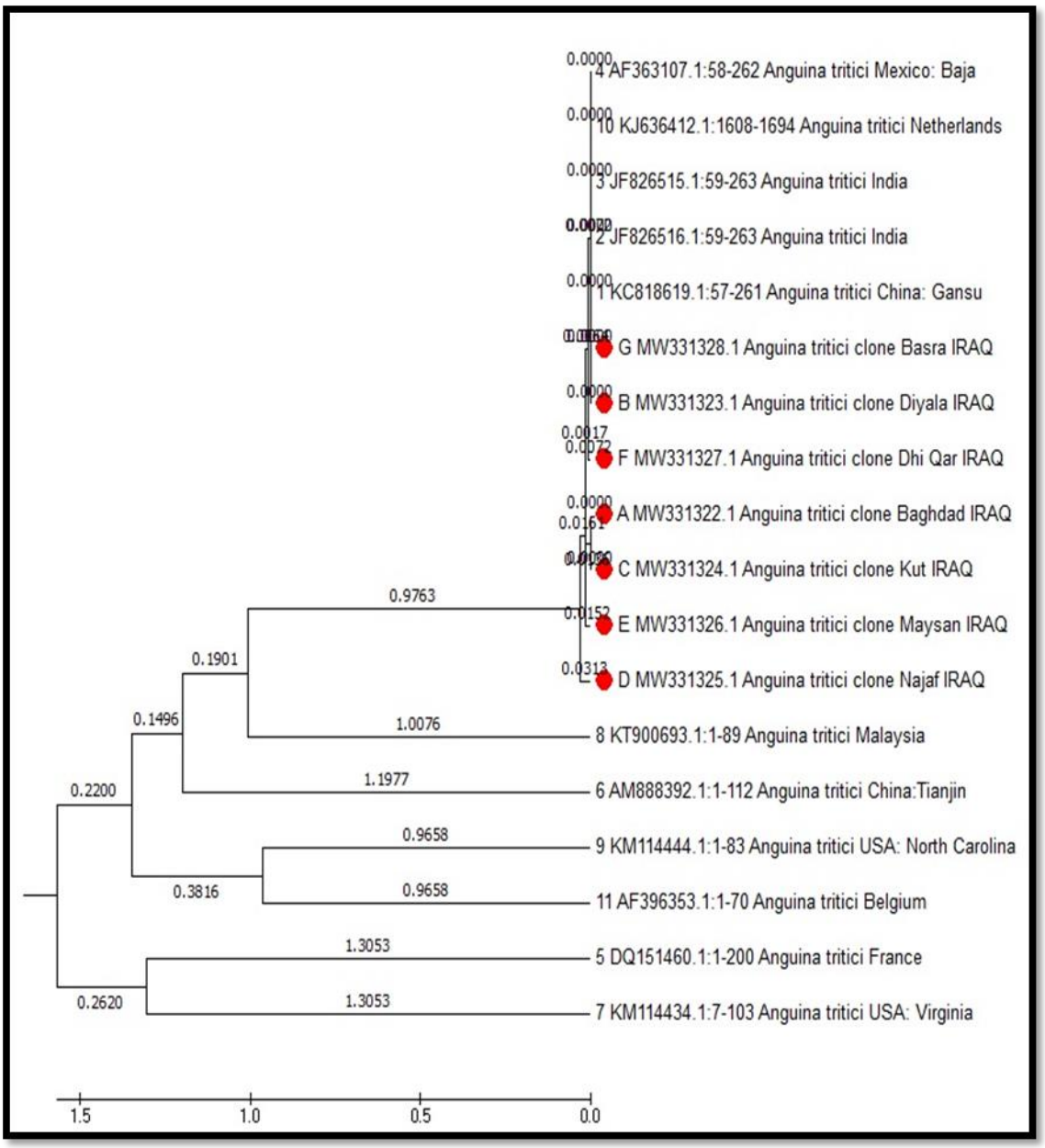

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Figure (4): Neighbor-joining tree *Anguina tritici* of *ITS* gene.

DISCUSSION

The research by [15] from Erbil and Duhok in the Kurdistan Region of Iraq showed the results indicating that all previous studies on the wheat seed gall nematode *A. tritici* in Iraq that assumed both durum wheat isolate and bread wheat isolate of that nematode to be different races are not accurate.

Although the two isolates are the same, the susceptibility of wheat cultivars, environmental factors, the timing of planting, farmer applications of pesticides and herbicides, and agricultural practices that in some way affect the pathogenicity of *A. tritici* are what cause durum or bread wheat to become infected by this nematode[16, 7].

For the first time, the molecular study by [15] highlighted that both nematode isolates from durum and bread wheat had the same genetic traits depending on the ampli-

fication results of PCR reactions. We also state that *A. tritici* has two races, including the wheat and barley races. The pathogenicity of the wheat race varies depending on the wheat cultivar, the nematode's behavior and the infection's intensity, which might vary in different regions and climatic conditions. In order to better clarify these nematode races in Iraq, new molecular research on various populations is being implemented in the current study of *A. tritici* in the southern and midland areas of the country.

CONCLUSIONS

The ITS genes obtained in this study were submitted and registered in the NCBI database (reference number for the samples MW331328.1/MW331327.1/MW331326.1/MW331323.1/MW331324.1/ MW331322.1/ MW331325.1).

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