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Sequencing Analysis of the ITS Region for Some *Fusarium* Species Isolated from Infected Tomato Plants

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Abstract

Fusarium wilt is one of the most important diseases that affects various plants and is caused by the fungus *Fusarium oxysporum. Fusarium* wilts in tomato plants are caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL), whether in fields or greenhouses. The tomato plant is considered one of the most important plants. This disease, however, affects its agricultural production. The current research aimed to diagnose *Fusarium* species that were isolated from infected tomato plants from different regions in Iraq by using molecular and morphological methods and to study the evolutionary relationship between the studied species, as twenty *Fusarium* isolates were obtained from infected tomato plants. Genomic DNA was amplified, using primers ITS1 and ITS4 to amplify ITS region, as there were differences between the results of the morphological diagnosis, which were based on the study of morphological features, and the molecular diagnosis which was based on the ITS region. Depending on the morphological features, F7 and F8 isolates were diagnosed as *F. oxysporum*, while the molecular diagnosis of the ITS regions showed that they were *F.incarnatum* and *F.proliferatum* respectively. For the rest of the isolates, the morphological and molecular diagnoses were consistent with *Fusarium oxysporum*. All isolates were recorded in the Gen Bank, and were given their accession numbers. An evolutionary tree was also made for the studied isolates to know the degree of genetic convergence and divergence between them. The current study concluded that the tomato plants in the field and greenhouse from different regions in Iraq were infected with different *Fusarium* species, with *Fusarium oxysporum* f. sp. *lycopersici,* being the most common species, was widely spreading among the identified isolates. Conventional methods based on morphological features take time and may result in incorrect identification of closely related species. Henceforth, *Fusarium* species may be correctly identified by using molecular methods based on ITS sequences.

Keywords: *Fusarium oxysporum*, Molecular method, ITS region, Phylogenetic relationship, Tomato plant

تحليل تسلسل منطقة ITS لبعض أنواع *Fusarium* المعزولة من نباتات الطماطم المصابة

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الخلاصة

يعد مرض ذبول الفيوزاريوم احد الأمراض المهمة التي تصيب النباتات المختلفة ويسببه فطر

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Fusarium oxysporum. المسبب لهذا المرض في نباتات الطماطم هو (Fusarium oxysporum f.
sp. lycopersici (FOL) سواء في الحقول أو البيوت الزجاجية. يعد نبات الطماطم من النباتات المهمة اذ يؤثر
هذا المرض على إنتاجه الزراعي. هدف البحث الحالي إلى تشخيص أنواع الفيوزاريوم التي تم عزلها من نباتات
الطماطم المصابة من مناطق مختلفة في العراق باستخدام الطرق الجزيئية والمظهرية ودراسة العلاقة التطورية
بين الأنواع المدروسة، اذ تم الحصول على عشرين عزلة Fusarium من نباتات الطماطم المصابة. تم تضخيم
الحمض النووي الجينومي باستخدام البادئات ITS1 و ITS4 لتضخيم منطقة الـ ITS ، اذ وجد هناك اختلافات
بين نتائج التشخيص المظهري الذي اعتمد على دراسة الصفات المظهرية والتشخيص الجزيئي الذي أعتمد على
منطقة الـ ITS– ، اعتمادًا على الصفات االمظهرية تم تشخيص العزلتين F7 و F8 على أنها Fusarium
oxysporum ، بينما أظهرالتشخيص الجزيئي لمناطق الـ ITS أنها Fusarium incarnatum و
Fusarium proliferatum على التوالي ، اما العزلات الباقية فقد تطابق التشخيص المظهري مع الجزيئي
بكونها Fusarium oxysporum . تم تسجيل جميع العزلات في بنك الجينات واعطيت ارقام خاصة بها ،
وقد تم ايضا عمل شجرة تطورية للعزلات المدروسة لمعرفة درجة التقارب والتباعد الوراثي فيما بينها. نستنتج
من الدراسة الحالية أن نباتات الطماطم في الحقل والبيوت الزجاجية من مناطق مختلفة في العراق مصابة بأنواع
مختلفة من الفيوزاريوم، وأكثر الأنواع شيوعاً هو Fusarium oxysporum f. sp. lycopersici، والذي انتشر
على نطاق واسع بين العزلات التي تم تشخيصها، أن الطرق التقليدية المعتمدة على دراسة الصفات المظهرية
تستغرق وقتًا وقد تؤدي إلى تشخيص غير دقيق للأنواع ذات الصلة الوثيقة؛ ونتيجة لذلك، يمكن تشخيص أنواع
الفيوزاريوم بشكل صحيح باستخدام الطرق الجزيئية القائمة على تسلسل ITS.

1. Introduction

The tomato (*Solanum lycopersicum* L.) is grown all over the world and used in processed foods and table cuisine [1]. It is one of the most significant genera of plant pathogenic fungi *Fusarium* and the most significant vegetable crop that is widely grown in Iraq. It has a history of severe infections in many economically significant plants, including tomato, eggplant, cucumber, etc. [2].

Fusarium wilt, induced by the soil-borne fungus, is one of the main biological factors that decrease the output of this crop [3]. One of the most significant and destructive diseases in the world is *Fusarium oxysporum* f.sp. *lycopersici* (Fol),[4], *F. oxysporum* is so prevalent in soils that it is now considered part of the global mycoflora. When a crop is grown, the pathogen can attack it because this fungus can survive latently for long periods in the soil in the form of spores [5].

The fungal diseases particularly wilt diseases and root rot caused by *F. oxysporum,* are the primary cause of the drop in tomato production in Iraq, resulting in the average of 40–50% loss of the yield [6]. *Fusarium* species are commonly identified by their macroscopic and microscopic characteristics [7]. Most reports, however,claim that these features are unstable,and, the most recommended method for diagnosing wilt disease is a microscopic examination of infected tissues and that of the pathogen based on morphological features, biochemical and allozyme characteristics, etc., which requires specialist knowledge and estimations that are subject to error, these time-consuming methods are insufficient and limiting [8]. The information required for a taxonomic purpose for identifying species as well as to better understand the evolutionary relationships among species is provided by molecular tools such as ITS and RAPD [9].

This implies the efficacy and utility of molecular approaches, enabling further characterization of fungi and other organisms [10]. This sequencing data has been widely studied in the classification and phylogenetic analysis of *Fusarium* species and its variability is primarily found in the introns providing sufficient resolution at the sub-species level [9].

In this current study, the morphological characteristics of *Fusarium* isolates were described and internal transcribed spacer region (ITS) was used to identify *Fusarium* species. The phylogenetic relatedness between the *Fusarium* isolates was also examined in this study.

2. Materials and Methods

❖ *Fungal Pathogen Isolation, Purification, and Diagnosis*

Infected tomato plants were collected during the period from August to December 2022 and January 2023 from fields and greenhouses in Al-Rashidiya, Al-Zaafaraniya, Al-Mahmoudiya, Al-Nahrawan , and Al-Taji (Latitude 33.3° N, Longitude 44.4° E) , Al-Swayrah (Latitude 32.5° N, Longitude 45°.8 E) , Karbala(Latitude 32°.6 N, Longitude 44° E) in Iraq. The number of tomato plants in each field ranged between 750-1000. The plants were placed in individual plastic bags, with the name of the location and the date of collection recorded on each bag. The infected plants in a field and greenhouse were recognized based on the symptoms that emerged on the shoots and roots, including leaves yellowing and drooping, death of some branches, reddish-brown streaks that could be seen in the vascular tissues of the stem when cut with a knife, and eventually, the death of plants (Figure 1). Infected plants' stems were cut off 15 cm above the area of the crown [11]. The pathogens were isolated and then identified from infected samples in the lab. The mud that had been suspended in the roots was removed after one hour of washing with tap water. The stems and roots were divided into small pieces (0.5–1 cm) and disinfected by submerging them for three minutes in a sodium hypochlorite solution (1% free chlorine), then rinsed three times with distilled sterilized water and then dried using sterile filter paper. For each infected plant, five portions (two for the root and three for the stem) were cultivated in Petri dishes containing autoclaved PDA and incubated for five days at 25 ±2°C [12]. To purify the fungi isolates, mycelia plugs with a 5mm diameter were taken from the growing margin onto the center of the petri dish containing new PDA where they were incubated for five days at 25 ±2°C. According to [7], morphology of the colony, conidiophores and spore forms were used to identify *Fusarium* isolates. All fungal isolates were cultivated and preserved on autoclaved PDA and incubated for five days at 25±2 °C. [3,6]. Moreover, slant cultures were created and kept in a refrigerator at 4°C.

A. In fields B. In greenhouses

Figure 1: The symptoms of *Fusarium* wilt on infected tomato plants

Pathogenicity Test

This test was carried out to detect the pathogenicity of *Fusarium* species isolates. Local tomato seeds untreated with fungicide were surface sterilized by immersing them in the sodium hypochlorite solution (2% free chlorine) for 2 minutes, then rinsing them three times with sterile distilled water and drying them with sterile filter paper. These fungal isolates were grown on PDA plates for 7 days. One plug (5mm) was then taken from each fungal isolate separately and

put in a test tube containing 10ml sterile distilled water then shaken well by hand (three minutes) to make fungal suspension. After that each 11 local tomato seeds (untreated with fungicide and surface sterilized by sodium hypochlorite) were soaked in each of the fungal suspensions (5ml) for 30 min separately, then inoculated seeds were placed on the sterile petri dish containing PDA and left for 7 days at 25±2°C. The percentage of infected seedlings was accounted according to the formula below (13):

$$P = \frac{\text{Number of non-germinating seeds}}{\text{Total number of seeds planted}} \times 100$$

P = The percentage of infected seedling

❖ *Genomic DNA Extraction*

Genomic DNA was extracted from twenty isolates of *Fusarium* sp. from infected tomato plants and named F1-F 20, according to the protocol of ABIO Pure Extraction. This kit was used to quickly and easily isolate DNA from hard-to-lyse fungus. All the isolates utilized in the DNA extraction were initially purified multiple times until any contamination (other fungus spores or bacteria) was removed by culturing the *Fusarium* isolates on the PDA medium for activation. The activation stage was crucial for DNA extraction to obtain pure cultures and a good product of genomic DNA [14].

❖ *Primers of Gene ITS*

The Macrogen Company provided these primers in lyophilized form. Lyophilized primers were dissolved in a stock solution of nuclease-free water to a final concentration of 100pmol/µl. To make a workable primer solution containing 10pmol/µl of these primers, 90µl of nuclease-free water was mixed with 10µ l of primer stock solution (stored at -20°C). The ITS sequences, which are found in all eukaryotes as a conserved region, were amplified using the universal primers (ITS-1 & ITS-4) [15, 16].

Table 1: The specific primer of gene ITS

Primer Name	Primer Sequence	Annealing Temp. (°C)	Product Size (bp)
ITS 1	5`-TCCGTAGGTGAACCTGCGG-3`	55	550 bp
ITS4	5`-TCCTCCGCTTATTGATATGC-3`		

❖ *PCR Amplification*

The PCR amplification was carried out using a thermal cycler in a total volume of 25µl consisting of 3µl DNA, 12.5 µl of master mix (Promega, USA), 1µl of each primer, and 7.5µl of nuclease-free distilled water. The 35 cycles of amplification were as follows: initial denaturation at 95 °C for 5 min; denaturation at 95 °C for 30 sec; annealing at 55 °C for 30 sec; first extension at 72 °C for 30 sec; and final extension at 72 °C for 7 min using a thermal cycler. All PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ultraviolet exposure.

❖ *Analysis of Sequence*

Using nucleotide BLAST in Gen Bank, the ITS gene sequences obtained in the present research were compared to other fungal isolate sequences retrieved from the NCBI database.

❖ *Phylogenetic Analysis of Fusarium Isolates*

The phylogenetic *analysis* was performed using the MEGA 6 program .The sequence analysis of twenty nucleotides was covered. First, second, third, and noncoding codon locations were included. All positions with gaps and missing data were eliminated. The total numbers of sites in the final dataset were 462.

❖ *Statistical Analysis:*

The Statistical Analysis System program was used to detect the effect of difference factors in study parameters (17). The least significant difference (LSD) test Analysis of Variance (ANOVA) was used to compare between means in this study.

3. Results and Discussion

3-1 -Morphological Identification of Fusarium Isolates

Twenty *Fusarium* isolates were obtained from 250 samples of infected tomato plants (Table 2) . Pathogenic *Fusarium* isolates were purified and identified by using morphological and microscopic characteristics. The morphological characteristics of *Fusarium* isolates were observed on CLA media, such as cottony mycelium growth and changing color according to age, from a colony with pinkish violet color to white to orange, white, and cream-colored colonies (Figure 2). Characteristics of *F.* oxysporum observed under a microscope revealed that macroconidia had sickle-shaped, three to five septate, and basal cells with a foot shape and their dimension ranged between 13.24-25.6µm in length and 2.35-4.78 µm in width. Microconidia were oval to in reniform, without septa and their dimension ranged between 7.44-12.48 µm in length and 2.14x4.81 µm in width, in addition to the chlamydospores, as is seen in culture. These features matched with *F. oxysporum* and were in concord to [18,19 , 20, 2

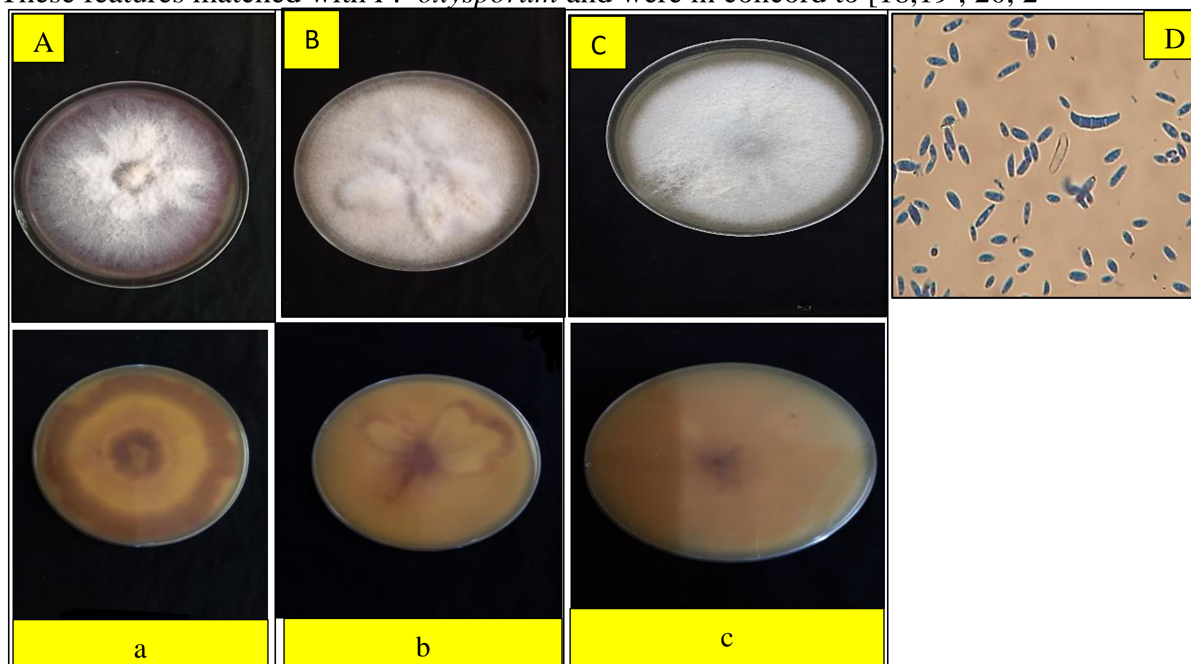


Figure 2. Morphological Identification of Fusarium isolates

(A) Fusarium oxysporum f. sp. lycopersici morphology on PDA medium (7days). (A): Front view of the plate (a): back view of the plate

(B) Fusarium proliferatum morphology on PDA medium (7days) (B): Front view of the plate (b): back view of the plate

(C) Fusarium incarnatum morphology on PDA medium at (7days) (C): Front view of the plate (c): back view of the plate

(D) Fusarium oxysporum f. sp. lycopersici morphology under microscope.

Table 2: Morphological characteristics of isolated *Fusarium* species.

No	*Fusarium* Species	Microconidia			Macroconidia			Mycelia Growth Rate Calculated (in MM/ Day)	Colony Shape	Colony Color	Sampling Tissue
		*Dimension			*Dimension						
		Length (Mm)	Width Mm)	Shape	Length (Mm)	Width (Mm)	Shape				
1	*Fusarium oxysporum* f.sp. *lycopersici*	7.79	2.33	Oval to Reniform, 0 septa	13.51	2.97	sickle-shaped ,3-5 Septa	7.4	Regular	White	Stem
2	*Fusarium oxysporum* f.sp. *lycopersici*	8.40	2.56	Oval to Reniform, 0 septa	13.24	3.69	sickle-shaped ,3-5 Septa	10.6	Regular	Red violet	Stem
3	*Fusarium oxysporum* f.sp. *lycopersici*	8.34	3.45	Oval to Reniform, 0 septa	14.36	3.75	sickle-shaped ,3-5 Septa	9.8	Regular	White	Crown
4	*Fusarium oxysporum* f.sp. *lycopersici*	8.59	3.35	Oval to Reniform, 0 septa	16.55	2.74	sickle-shaped ,3-5 Septa	8.9	Regular	White	Stem
5	*Fusarium oxysporum* f.sp. *lycopersici*	7.69	2.14	Oval to Reniform, 0 septa	22.53	3.84	sickle-shaped ,3-5 Septa	9.3	Regular	White	Stem
6	*Fusarium oxysporum* f.sp. *lycopersici*	10.35	4.13	Oval to Reniform, 0 septa	13.62	4.78	sickle-shaped ,3-5 Septa	8.9	Regular	Pale white	Stem
7	*Fusarium incarnatum*	7.98	3.93	oval	17.59	3.25	Curved with ,3Septa	10.8	Serrated	White orange	Stem
8	*Fusarium proliferatum*	7.44	3.25	oval	14.9	3.82	Slender to relative straight ,3-5 Septa	11.2	regular	White	Stem
9	*Fusarium oxysporum* f.sp. *lycopersici*	9.23	3.43	Oval to Reniform, 0 septa	20.32	2.55	sickle-shaped ,3-5 Septa	8.8	Serrated	White pigmented violet	Stem
10	*Fusarium oxysporum* f.sp. *lycopersici*	8.76	2.78	Oval to Reniform, 0 septa	25.6	3.56	sickle-shaped ,3-5 Septa	11.6	regular	White orange	Stem
11	*Fusarium oxysporum* f.sp. *lycopersici*	7.98	3.76	Oval to Reniform, 0 septa	16.89	2.35	sickle-shaped ,3-5 Septa	7.9	regular	White	Stem
12	*Fusarium oxysporum* f.sp. *lycopersici*	8.54	2.98	Oval to Reniform, 0 septa	23.55	3.38	sickle-shaped ,3-5 Septa	7.6	regular	White	Stem
13	*Fusarium oxysporum* f.sp. *lycopersici*	10.48	3.25	Oval to Reniform, 0 septa	19.88	3.59	sickle-shaped ,3-5 Septa	9.4	Serrated	Red violet	Stem
14	*Fusarium oxysporum* f.sp. *lycopersici*	10.13	3.49	Oval to Reniform, 0 septa	20.45	3.43	sickle-shaped ,3-5 Septa	9.7	Serrated	Violet	Root
15	*Fusarium oxysporum* f.sp. *lycopersici*	9.12	3.14	Oval to Reniform, 0 septa	18.89	3.84	sickle-shaped ,3-5 Septa	10.4	Serrated	White pigmented pale violet	Stem
16	*Fusarium oxysporum* f.sp. *lycopersici*	8.23	4.25	Oval to Reniform, 0 septa	21.19	3.26	sickle-shaped ,3-5 Septa	9.9	serrated	White pigmented violet	crown
17	*Fusarium oxysporum* f.sp. *lycopersici*	10.57	3.98	Oval to Reniform, 0 septa	25.33	3.98	sickle-shaped ,3-5 Septa	9.7	Serrated	White	Stem
18	*Fusarium oxysporum* f.sp. *lycopersici*	9.35	2.98	Oval to Reniform, 0 septa	13.89	2.93	sickle-shaped ,3-5 Septa	10.9	regular	Violet	Stem
19	*Fusarium oxysporum* f.sp. *lycopersici*	8.94	4.81	Oval to Reniform, 0 septa	17.65	3.15	sickle-shaped ,3-5 Septa	10.7	Serrated	White pigmented violet	crown
20	*Fusarium oxysporum* f.sp. *lycopersici*	12.48	2.96	Oval to Reniform, 0 septa	23.56	3.74	sickle-shaped ,3-5 Septa	10.2	Serrated	White	crown
LSD value	---	-----	----	---	------	----	---	1.154 *	---	---	---

***** ***(P≤0.05).***

*Mean of 10 spores from 2 microscopic fields

3 -2 -Pathogenicity Test of Fusarium Species

Results of the pathogenicity test showed that all *Fusarium* species isolates were highly pathogenic for local tomato seeds, and five of the *F. oxysporum* isolates (numbered F3, F 5, F9, F17 and F20) showed high pathogenicity more than other isolates which recorded 100% inhibition growth of local tomato seeds. The parasitic nature of the fungus which attacks the host's seeds and either causes them to rot or prevents them from germinating by secreting poisonous substances that kill the embryos and multiple enzymes that break down cellulose, chitin, and protein which causes the seeds to rot, is what has caused the increase in the proportion of rotting seeds (Table 3) [22]. Findings that all *Fusarium* isolates exhibited highly significant virulence against cress seeds and reached high pathogenicity confirmed our findings.

Table 3: Pathogenicity test of *Fusarium* species on local tomato seeds

N. of Isolates	*Fusarium* Species	Mean ± SE
1	*Fusarium oxysporum* f.sp. *lycopersici*	98.67 ±0.33
2	*Fusarium oxysporum* f.sp. *lycopersici*	95.33 ±1.45
3	*Fusarium oxysporum* f.sp. *lycopersici*	100 ±0.00
4	*Fusarium oxysporum* f.sp. *lycopersici*	99.33 ±0.67
5	*Fusarium oxysporum* f.sp. *lycopersici*	100 ±0.00
6	*Fusarium oxysporum* f.sp. *lycopersici*	97.33 ±1.45
7	*Fusarium incarnatum*	90.33 ±145
8	*Fusarium proliferatum*	94.67 ±2.33
9	*Fusarium oxysporum* f.sp. *lycopersici*	100 ±0.00
10	*Fusarium oxysporum* f.sp. *lycopersici*	98.33 ±0.88
11	*Fusarium oxysporum* f.sp. *lycopersici*	97.33 ±1.20
12	*Fusarium oxysporum* f.sp. *lycopersici*	98.00 ±1.00
13	*Fusarium oxysporum* f.sp. *lycopersici*	96.67 ±0.88
14	*Fusarium oxysporum* f.sp. *lycopersici*	99.00 ±0.57
15	*Fusarium oxysporum* f.sp. *lycopersici*	98.67 ±0.33
16	*Fusarium oxysporum* f.sp. *lycopersici*	99.33 ±0.33
17	*Fusarium oxysporum* f.sp. *lycopersici*	100.00 ±0.00
18	*Fusarium oxysporum* f.sp. *lycopersici*	99.33 ±0.67
19	*Fusarium oxysporum* f.sp. *lycopersici*	98.00 ±0.57
20	*Fusarium oxysporum* f.sp. *lycopersici*	100 ±0.00
LSD value		2.686 *

***** *(P≤0.05).*

3 -3 -Sequencing Analysis of ITS Region

The amplified product likewise produced a single band in electrophoresis at 550 - 570 bp for the 20 samples of *Fusarium* isolates following the PCR run (Figure 3). All 20 isolates' ITS sequences were presented to the database in the NCBI Gen Bank to obtain their accession numbers (Table 4). The nucleotide identity of the isolates ranged from 99–100% with *Fusarium* isolates, according to an analysis of the BLAST for ITS sequences. The analysis of sequencing was carried out by using Geneious software, and it was found that the nucleotide sequence was different from that of other internationally known genes. They were insertion, transversion, and transition. These alterations are detailed in Table 4. The current study successfully confirmed that the ITS region was appropriate for providing target genes for the

molecular identification of *Fusarium* spp. isolates. Variation in the nucleotide sequences of the ITS region was successfully used to distinguish among the samples.

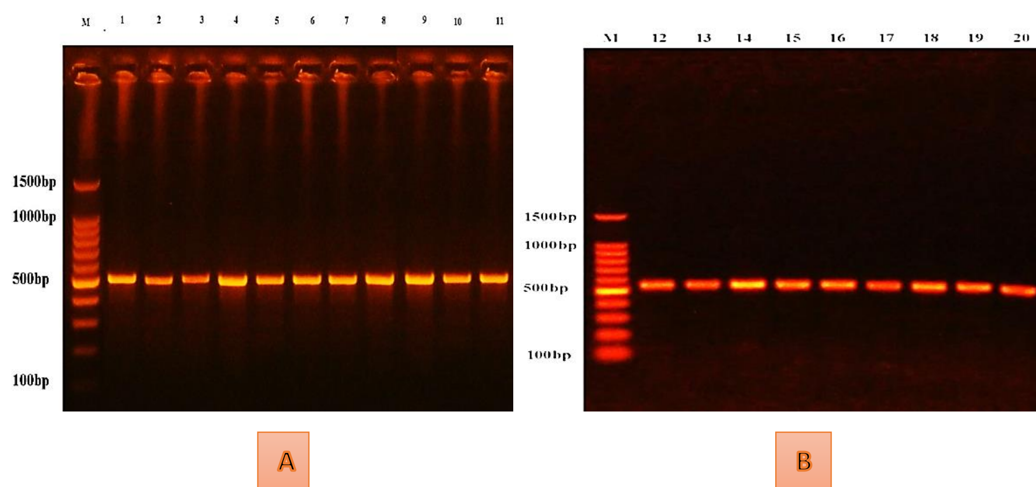


Figure 3 (A &B): Results of the amplification of *ITS* gene of *Fusarium* species were fractionated on 1.5% agarose gel electrophoresis stained with Ethedium Bromide M: (100 – 1500) bp DNA ladder marker. Lanes 1-20 resemble 550 - 570bp PCR Products, (Lanes 1-20 represent number of samples F1 –F20).PCR product of ITS gene band size 550bp that was electrophoresis on 1.5 % agarose gel ITS gene.

Table 4: Genetic variation small subunit of ribosomal RNA Gene (ITS region)28S ribosomal RNA gene

No.	Type of Substitution	Location	Nucleotide	Sequence ID with Comparisone	Sequence ID with Submission	Source	Identities
1	**Transversion** **Insertion**	280 -436	C\G -\G	**ID: KF914464.1**	**ID: OQ439282.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
2	**Transversion**	234	A\C	**ID: MW927137.1**	**ID: OQ439283.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
3	**Transition** **Transversion**	300 381	T\C C\G	**ID: MW927137.1**	**ID: OQ439284.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
4	**Transition** **Transition**	300 389	T\C C\T	**ID: MW927137.1**	**ID: OQ439285.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
5	**Transition** **Transversion**	300 538	T\C A\T	**ID: MW927137.1**	**ID: OQ439286.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
6	**Transition**	300	T\C	**ID: MW927137.1**	**ID: OQ439287.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
7	-----------	------	-------	**ID: OP006283.1**	**ID: OQ439288.1**	***Fusarium incarnatum***	**100%**
8	**Insertion**	-550	-\T	**ID: MW426274.1**	**ID: OQ439289.1**	***Fusarium proliferatum***	**99%**
9	**Transversion**	459	T\A	**ID: OP750472.1**	**ID: OQ439290.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
10	**Transversion** **Transversion** **Transversion** **Transversion** **Transversion**	176 181 362 423 432	T\A C\G C\A A\T A\T	**ID: OP750472.1**	**ID: OQ439291.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
11	**Insertion** **Transversion** **Transversion** **Insertion**	-84 176 181 -494	-\C T\A C\G -\C	**ID: OP750472.1**	**ID: OQ439292.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**

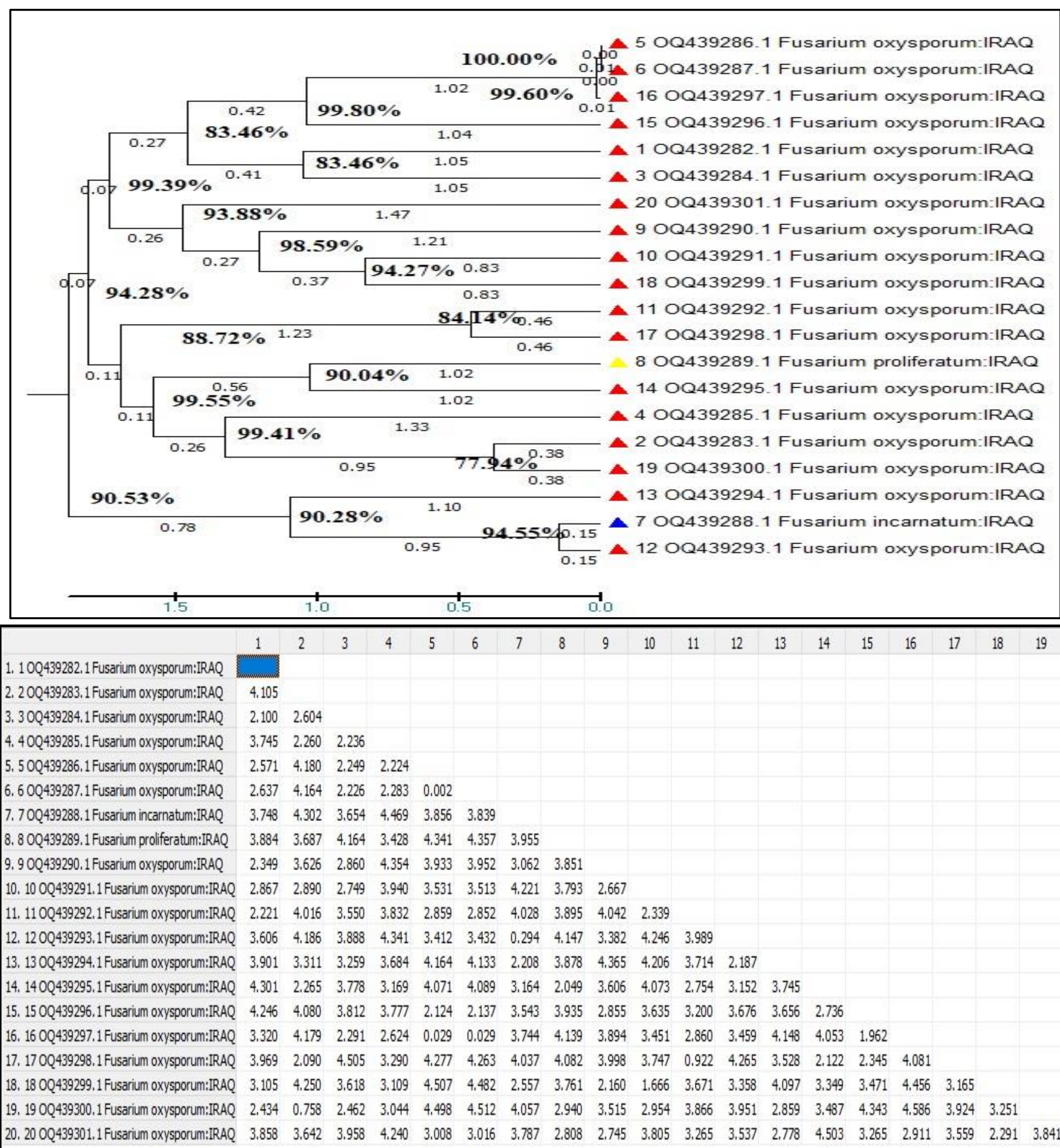
12	**Transition**	350	G\A	**ID: MW927137.1**	**ID: OQ439293.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
13	-----------	------	-------	**ID: OP750477.1**	**ID: OQ439294.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**100%**
14	-----------	------	-------	**ID: MW927137.1**	**ID: OQ439295.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**100%**
15	-----------	------	-------	**ID: MW927137.1**	**ID: OQ439296.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**100%**
16	-----------	------	-------	**ID: MK917791.1**	**ID: OQ439297.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**100%**
17	**Insertion**	-356	-\C	**ID: KF914464.1**	**ID: OQ439298.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
	Transversion	471	C\G				
	Transition	472	G\A				
	Transversion	482	G\T				
	Transversion	500	A\T				
	Transversion	507	T\G				
18	**Transition**	300	T\C	**ID: MW927137.1**	**ID: OQ439299.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
	Transition	389	C\T				
	Transversion	538	A\T				
19	-----------	------	-------	**ID: MN071397.1**	**ID: OQ439300.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**100%**
20	**Insertion**	-170	-\A	**ID: MH855098.1**	**ID: OQ439301.1**	***Fusarium oxysporum* f. sp. *lycopersici***	**99%**
	Insertion	-172	-\A				

The ribosomal RNA gene of *Fusarium* species isolates results obtained showed that F1 isolate numbered OQ439282.1 was similar at 99% to *Fusarium oxysporum* f. sp. *lycopersici*, including transversion mutation at C\G and insertion at -\G in the ribosomal RNA gene, while that F2 isolate OQ439283.1 was similar at 99% to *F. oxysporum* f. sp. *lycopersici*, included transversion mutation only at A\C in the ribosomal RNA gene. The F3 isolate OQ439284.1 was similar at 99% to *F. oxysporum* f. sp. *lycopersici* and contained transition at T\C and transversion mutations at C\G in the ribosomal RNA gene, while the F4 isolate OQ439285.1 was similar at 99% to *F. oxysporum* f. sp. *lycopersici,* showed transition mutation only at T/C and C/T in the ribosomal RNA gene. The F5 isolate OQ439286.1 was similar at 99% to *F. oxysporum* f. sp. *lycopersici* contained transversion at T/C and transition mutations at A/T in ribosomal RNA gene, while the F6 isolate OQ439287.1 was similar at 99% to *F. oxysporum* f. sp. *lycopersici*, included transition mutation only at T\C in the ribosomal RNA gene. The F7 isolate OQ439288.1 was 100% identical to *Fusarium incarnatum*, while the F8 isolate OQ439289.1 was 99% similar to *Fusarium proliferatum*, included insertion mutation only at -\T in the ribosomal RNA gene. The F9 isolate OQ439290.1 was 99% similar to *F. oxysporum* f. sp. *lycopersici* contained transversion mutation only at T/A in the ribosomal RNA gene, while the F10 isolate OQ439291.1 was 99% similar to *F. oxysporum* f. sp. *lycopersici* contained transversion mutation in five locations T\A, C\G, C\A, A\T & A\T in the ribosomal RNA gene. The F11 isolate OQ439292.1 was similar at 99% to *F. oxysporum* f. sp. *lycopersici* contained transversion at T\A& C\G and insertion mutations at -\C in the ribosomal RNA, while the F12 isolate OQ439293.1 was 99% similar to *F. oxysporum* f. sp. *lycopersici* contained transition mutation in G\A in the ribosomal RNA gene. The *Fusarium* isolates F13, F14, F15, F16 and F19 numbered OQ439294.1, OQ439295.1, OQ439296.1, OQ439297.1 and OQ439300.1 respectively were identical 100% to *F. oxysporum* f. sp. *lycopersici* in the ribosomal RNA gene. The F17 isolate OQ439298.1 contained insertion mutation at -\C and transversion mutation at C\G, G\T, A\T& T\G and transition at G\A with 99% similarity to *F. oxysporum* f. sp. *lycopersici* in the ribosomal RNA gene, while the F18 isolate OQ439299.1 contained transition at T\C& C\T and transversion mutation at A\T with 99% similarity to *F. oxysporum*

f. sp. *lycopersici* in ribosomal RNA gene. The F20 isolate OQ439301.1 was similar at 99% to *F. oxysporum* f. sp. *lycopersici* contained insertion mutation only at -\A (Table 4). In the current study based on morphological characteristics, the *Fusarium* isolates F7 and F8 were determined to be *F. oxysporum*. It was, however, very different with the ITS identification; F7 was determined to be *F. incarnatum* and F8 to be *F. proliferatum* based on the ITS region. The most common methods for identifying plant pathogenic fungi are biochemical, chemical and immunological testing, as well as selective media that promote their growth. Additionally, the morphological recognition for these fungi on nonselective media takes time and needs experienced taxonomists. The selective media may help in recognition up to the genus level but can't distinguish between various species [16,20]. Studies and research have shown that many pathogenic fungi, whether present in the soil or pathogenic to plants, can now be detected and identified by using molecular biology techniques, particularly PCR [23, 24]. Analysis of sequence is a better option for phylogenetic research in the *Fusarium* species because the ITS region is more frequently examined due to the region's species specificity and because it is known to provide better resolution at the sub-species level [9,25].

3 -4 Phylogenic Analysis of Fusarium Species(Figure 4 ,5,and 6)

The phylogenetic tree of *Fusarium* isolates were divided into three groups: the first group shared 94.28% similarity with the second group, while the first and second groups shared 90.53% similarity with the third group. The first group was divided into two branches; the first branch contained F5 which was found to be similar to F6 at 100% homology, but the F5 and F6 were found to be similar to F16 at 99.60% homology. However, the F5, F6, and F16 were found to be similar to F15 at 99.80% homology, and F1 was found to be similar to F3 at 83.46% homology. The second branch in the first group, F10, was found to be similar to F18 at 94.27% homology, while F10 and F18 were found to be similar to F9 at 98.59% homology. However, F9, F10, F9, F10, and F18 were found to be similar to F20 at 93.88% homology. As for the second group, it was divided into two branches. The first branch of it contained F17 which was similar to F11with a homology 84.14%. But the second branch in the second group contained F14 which was found to be similar to F8 with a homology 90.04%, while the isolate F19 was found to be similar to F2 with a homology 77.94%. F2 and F19 were found to be similar, with F4 showing 99.41% homology. As for the third group, it was divided into two branches; the second branch contained isolate F12 which was found to be similar to F7 with a homology 94.55%, while the first branch contained isolate F13 which was found to be similar to F12 and F7 with a homology 92.58%. This difference between isolates could be due to the different geographical regions from which fungal isolates were collected [26]. This implied the efficiency and benefit of molecular approaches for the identification of fungi and other organisms [21, 25]. Thus, there are shortcomings in morphological characterization and classical taxonomy for the recognition of species in the genus *Fusarium*, the information needed for taxonomic purposes, such as species identification and understanding the evolutionary relationships between species, can be obtained by molecular techniques like the ITS region [9, 22, 25].



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. 1 OQ439282.1 Fusarium oxysporum:IRAQ																			
2. 2 OQ439283.1 Fusarium oxysporum:IRAQ	4.105																		
3. 3 OQ439284.1 Fusarium oxysporum:IRAQ	2.100	2.604																	
4. 4 OQ439285.1 Fusarium oxysporum:IRAQ	3.745	2.260	2.236																
5. 5 OQ439286.1 Fusarium oxysporum:IRAQ	2.571	4.180	2.249	2.224															
6. 6 OQ439287.1 Fusarium oxysporum:IRAQ	2.637	4.164	2.226	2.283	0.002														
7. 7 OQ439288.1 Fusarium incarnatum:IRAQ	3.748	4.302	3.654	4.469	3.856	3.839													
8. 8 OQ439289.1 Fusarium proliferatum:IRAQ	3.884	3.687	4.164	3.428	4.341	4.357	3.955												
9. 9 OQ439290.1 Fusarium oxysporum:IRAQ	2.349	3.626	2.860	4.354	3.933	3.952	3.062	3.851											
10. 10 OQ439291.1 Fusarium oxysporum:IRAQ	2.867	2.890	2.749	3.940	3.531	3.513	4.221	3.793	2.667										
11. 11 OQ439292.1 Fusarium oxysporum:IRAQ	2.221	4.016	3.550	3.832	2.859	2.852	4.028	3.895	4.042	2.339									
12. 12 OQ439293.1 Fusarium oxysporum:IRAQ	3.606	4.186	3.888	4.341	3.412	3.432	0.294	4.147	3.382	4.246	3.989								
13. 13 OQ439294.1 Fusarium oxysporum:IRAQ	3.901	3.311	3.259	3.684	4.164	4.133	2.208	3.878	4.365	4.206	3.714	2.187							
14. 14 OQ439295.1 Fusarium oxysporum:IRAQ	4.301	2.265	3.778	3.169	4.071	4.089	3.164	2.049	3.606	4.073	2.754	3.152	3.745						
15. 15 OQ439296.1 Fusarium oxysporum:IRAQ	4.246	4.080	3.812	3.777	2.124	2.137	3.543	3.935	2.855	3.635	3.200	3.676	3.656	2.736					
16. 16 OQ439297.1 Fusarium oxysporum:IRAQ	3.320	4.179	2.291	2.624	0.029	0.029	3.744	4.139	3.894	3.451	2.860	3.459	4.148	4.053	1.962				
17. 17 OQ439298.1 Fusarium oxysporum:IRAQ	3.969	2.090	4.505	3.290	4.277	4.263	4.037	4.082	3.998	3.747	0.922	4.265	3.528	2.122	2.345	4.081			
18. 18 OQ439299.1 Fusarium oxysporum:IRAQ	3.105	4.250	3.618	3.109	4.507	4.482	2.557	3.761	2.160	1.666	3.671	3.358	4.097	3.349	3.471	4.456	3.165		
19. 19 OQ439300.1 Fusarium oxysporum:IRAQ	2.434	0.758	2.462	3.044	4.498	4.512	4.057	2.940	3.515	2.954	3.866	3.951	2.859	3.487	4.343	4.586	3.924	3.251	
20. 20 OQ439301.1 Fusarium oxysporum:IRAQ	3.858	3.642	3.958	4.240	3.008	3.016	3.787	2.808	2.745	3.805	3.265	3.537	2.778	4.503	3.265	2.911	3.559	2.291	3.841

Figure 4: The phylogenetic tree generated using ITS region nucleotide sequence information of *Fusarium* species (Iraqi isolates).

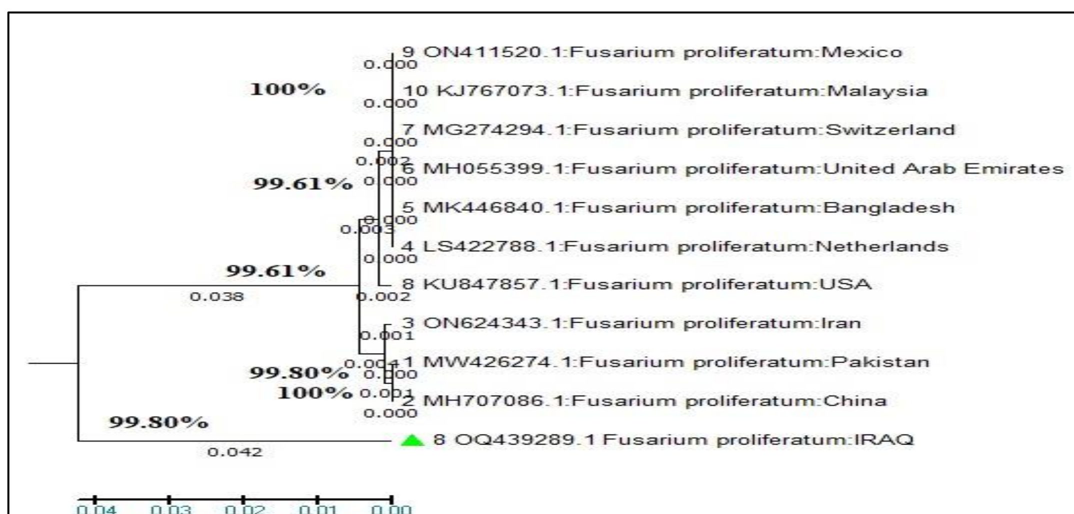


Figure 5: The phylogenetic tree generated using ITS region nucleotide sequence information of *Fusarium oxsporum* (Iraqi isolates in this study with other global isolates)

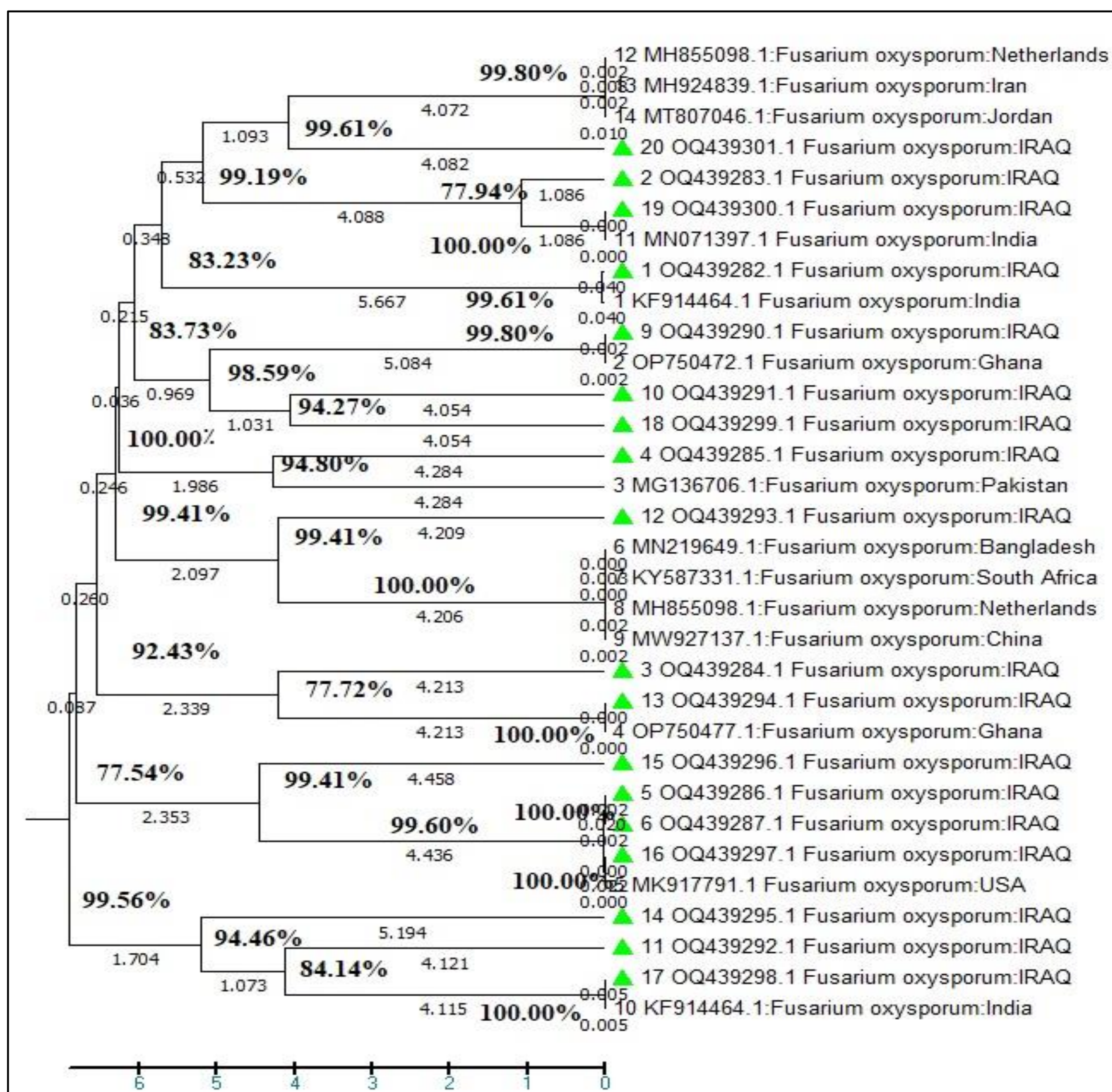


Figure 6: The phylogenetic tree generated using ITS region nucleotide sequence information of *Fusarium proliferatum* (Iraqi isolates in this study with other global isolates)

4 Conclusion

The current study concluded that the tomato plants in the field and greenhouse from seven different regions in Iraq were infected with different *Fusarium* species. *Fusarium oxysporum* f. sp. *lycopersici* the most common species, spreading widely among identified isolates. Conventional methods based on morphological features take time and could result in incorrect identification of closely related species. Hence, *Fusarium* species may be correctly identified by using molecular methods based on ITS sequences. A molecular tool like ITS can provide the necessary information required for a taxonomic purpose like species identification as well as to elucidate the evolutionary relationships among species.

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