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Molecular Detection of *Fusarium* species infected Corn in Kurdistan region-Iraq

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ABSTRACT

Corn or Maize product is regarded as one of the essential products in the world and stands third product after the rice and wheat crops. Different fungal pathogens attack corn plants; one of them is ear rot, brought on by *Fusarium* species and whose occurrence is primarily influenced by environmental factors. In order to isolate and identify *Fusarium* species from corn plants and their prevalence, 50 samples of corn were collected during September, October, and November of 2021 from 30 corn fields in 14 regions of different places in the Kurdistan region of Iraq. From all samples, 39 isolates of *Fusarium* were detected and based on morphological characteristics, six other species of *Fusarium* were identified, namely *F. verticillioides* (33.34%), *F. proliferatum* (25.64%), *F. oxysporum* (12.82%), *F. incarnatum* and *F. equiseti* (10.25% each), then *F. fujikuroi* (7.7%). The most prevalent species was *F. verticillioides* which was isolated from seven corn fields and significantly higher than all other isolated species. All *Fusarium* isolates were also molecularly identified depending on amplifying the internal transcribed spacer (ITS) universal region using forward ITS1 and reverse ITS4 primers and indicated DNA fragments ranged from 550 to 570 bp. The PCR fragments of the amplified ITS region were sequenced, aligned and registered in NCBI GeneBank with specified accession numbers. The phylogenetic tree and all analyzes were performed using the MEGA program version 11.0.13. The current study concluded that the corn fields in the Kurdistan region are infected with different *Fusarium* species, and the most common species is *F. verticillioides*. As well as the *Fusarium* species in the Kurdistan region have close evolutionary history to the same species in other countries. Thus, the study recommends more research to investigate the occurrence of toxigenic *Fusarium* species associated with cereal grains in the region.

Keywords: Corn Ear and Kernel Rot, Fusarium Species, ITS, Molecular Identification, And Phylogenetic Analysis.

1. Introduction

Corn or Maize product is regarded as one of the essential products in the world[1], and stands third product after the rice and wheat crops[2,3]. Maize is the only crop that can to flourish in such a wide range of environments. It can flourish anywhere from 58 north to 40 south, below sea level to more than 3000 m above it, and with rainfall ranging from 250 mm to 5000 mm annually[4]. Also corn is used to producing food for human and animal feeding, in many manufacturing industries. As well as corn may grow in a variety of climatic regions around the world, known as variable yields[5,6]. Corn is separated from different grain products with the rate of carotenoids and its content of oils, starch, and protein content, which gives it high nutritional content for animal and human infection related[4,5,6]. Different fungal pathogens attack maize plant, one of them is ear rot, which is brought on by *Fusarium* species and whose occurrence is primarily influenced by environmental factors like temperature, relative humidity, carbon dioxide, oxygen, substrate composition, and occasional rainfall, as well as agronomic factors like the use of pesticides and the susceptibility of particular plant varieties[7,8,9], seasonal weather, the genotype of the host, and insect activity are only a few of the variables that might affect the infection of corn grain by fungal pathogens on the farm. Because they are linked to stress conditions during plant flowering and kernel drying[10, 13], dates for planting and harvesting impact grain quality. Fumonisins accumulation and kernel infection by *Fusarium verticillioides* are among the most prevalent occur in the field, and both of these occurrences are significantly influenced by the environment[11,12]. At 30 °C or lower, *Fusarium* species increase in maize grains with a minimum moisture range of 20%, as shown in Figure 1, but at 37 °C, they became impossible[16]. So, in "Ethiopia" and "China," where many *Fusarium* species are common, maize diseases are common too[14,15].

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Figure 1: Corns infected with *Fusarium* species (A: Corn Farm, B: Aborted Kernel, C: Fungal Infection).

Moreover, *Fusarium* species infecting roots, stems, leaves, and grains with agricultural yields has drastically decreased, generally ranging from 15% to 35%. Some strains can also produce mycotoxins, harming plants infected before harvest[16,17]. Following it, Mycotoxin-tainted corn eating may result in a range of serious harmful effects in both animals and humans[14,5]. *Fusarium* species infect the maize during the middle of June and at the beginning of August when the maize is planted again for the second time[21]; maybe the *Fusarium* species return and cause plant disease in some areas according to temperature, especially when temperature decreases during fall on September[22]. Mycotoxin is one of the most significant issues with maize fielding infection[23], which are hazardous second-generation metabolites produced by certain fungi in the field and/or during storage[21,22]. Many mycotoxins, such as fumonisins produced by *Fusarium verticillioides*, *Fusarium proliferatum* or other *Fusarium* species, are not affected by heat and are heat stable[23,2]. Therefore, the only way to stop or lessen their influence is to prevent or reduce their production in the field. The precise recognition of the individual diseases and identification of their presence in sensitive crops offer crucial background data on the pathogen prevalence in the harvest. Also, standard evaluation determines the frequency of the regulating genes that control the mycotoxins biosynthetic pathway in many strains of *Fusarium*. May the measurement of levels of the associated mycotoxins is an equally significant piece of information[27]. The interplay between environmental stress factors such as water activity and temperature impacts the proliferation of toxigenic fungus species, expression of biosynthetic regulatory genes, and the production of mycotoxin[28]. Fusarium is a major pathogenic genera affecting corn, mainly due to F. verticillioides; F. proliferatum, F. fujikuroi, F. oxysporum, F. equiseti, F. incarnatum, and another species, there are several of this Fusarium in soils. The following are the infection pathways: Fusarium from infected seeds can spread to seedlings; conidia from water splashed on plants or carried by the wind may land on the silk and then reach the kernels; infection spreading from kernels to cob can continue through the stalk; infection spreading through root and stalk can spread systemically and reach the ear and kernel rot[26-28]. This study aims to isolate and identify *Fusarium* species from corn plants and their prevalence in some fields of the Kurdistan region of Iraq.

2. Methods and Materials

2.1 Collection of Samples

In total, 50 samples of corn were collected during September, October, and November of 2021 from 30 corn fields in 14 regions of different places in the Kurdistan region of Iraq, as shown in figure 2 and table 1. Samples were not collected in Duhok, Sulaimani and Koya district due to the lack of corn fields during the growing season. The samples included ear and kernel rot of corn during the ever-increasing seasons[29,30]. Corn samples were collected within field randomly and transferred to the lab, then stored in the refrigerator until the culture media preparations.

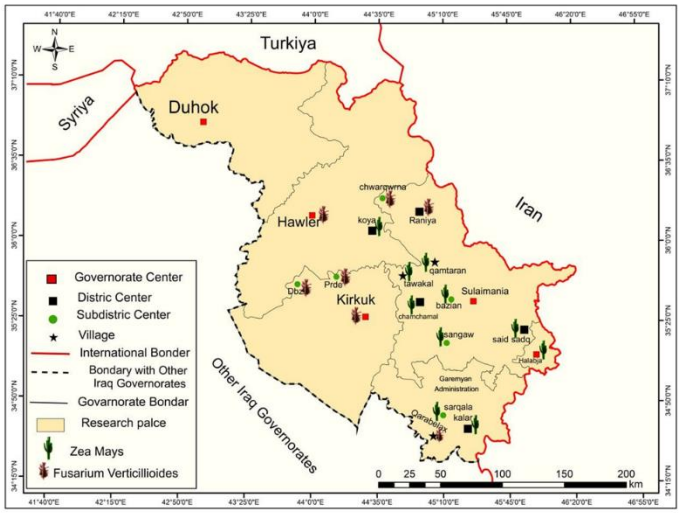

Figure 2: The map of corn fields where samples are collected.

Table 1: Number of samples and fields from each collection region of Kurdistan-Iraq.

Region	Number of fields	Number of samples
Hawler	2	6
Kirkuk	5	8
Kalar	2	4
Prde	8	9
Bazean	1	1
Dbs	3	5
Ranya	1	3
Chwarqurna	1	2
Halabja	2	2
Said sadiq	1	2
Chamchamal	1	3
Sangaw	1	2
Sarqala	1	1
Qarabelax	1	2
Total	**30**	**50**

2.2 Isolation of Fungal Cultures

Suspected samples of corn kernels with naked eyes were used for fungal isolation. Using a sterilized needle, a part of the infected corn grain was transferred to Potato Dextrose Agar (PDA) and incubated for 5-7 days under 26°C±1. The second technique of fungal isolation was carried out using the dilution method for those samples where the fungal infection was not seen with the naked eye. The corn kernel was ground, and 10gm of corn was added to 100 mL of ddH_2O and shaken for 5 minutes using a shaker device[34]. Then 1ml of stock solution was transferred to 9ml of ddH_2O and mixed with vortex for 1 minute. Then 1mL of

the second solution was transferred to 9ml of ddH2O and mixed with vortex for 1 minute. Then 1ml of each first and second dilution was transferred to PDA and streaked using L-shape glass as shown in Figure 3. The plates were incubated for 5-7 dates under 26°C±1[14,9].

The grown fungal colonies on PDA were subcultured and incubated for 5-7 days under the same temperature to get pure fungal cultures. Then, after, pure cultures were used for further macroscopic and microscopic examinations.

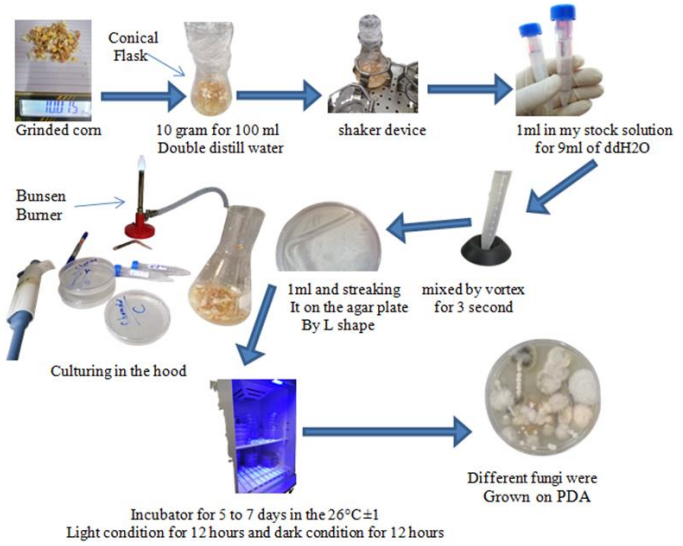

Figure 3: The dilution method technique for fungal isolation.

2.3 Staining by Lacto Phenol Cotton Blue

Pure cultures were used for macroscopic observations, as well as used for microscopic observations with the medium mounting technique. A drop of lactophenol blue solution was placed on a glass slide. Then a part of the fungal colony from the margin was removed using a sterile inoculation loop and then transferred to the slide. A coverslip was gently placed on the slide to avoid air bubbles, and then examined under 10x and 40x of magnification power. A list of macroscopic features, such as colony type, above colour, reverse colour, and microscopic features, including hyphae, chlamydospores, macroconidia, and microconidia, were registered for each isolate[35].

2.4 DNA Extractions of *Fusarium* Species

DNA was extracted using EasyPure Plant Genomic DNA Kit (South Korea) from the isolates of *Fusarium* species, which identified with the classical method. The mycelia of *Fusarium* isolates grown for 5-7 days on a PDA medium were collected and ground under liquid nitrogen.

2.5 Molecular Identification of *Fusarium* species using ITS region.

Fusarium isolates are molecularly identified based on the ITS universal region, which is the region located between the tiny nuclear 18S rDNA and large nuclear 28S rDNA, including 5.8S rDNA. The primers **ITS1** (5-3) Forward (TCCGTAGGTGAACCTGCG) and **ITS4** (5-3) Reverse (TCCTCCGCTTATTGATATGC) were used to amplify the ITS region, which indicated by PCR fragments ranged from 550-570 bp. To prepare 30 µl of the sample, the PCR tube containing 10 µl PCR master mix [Taq Master (2x conc.) / add a bio. South Korea] and 6 µl of DNA sample, primers (ITS1) forward 1.5 µl, (ITS4) reverse 1.5 µl, and 11 µl ddH_2O. PCR was performed on a Thermal (Mega) cycler[34]. The PCR program was as follows: one cycle of Initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, extension at 72°C for 1 min, then final extension at 72°C for 10 min[36].

2.6 Gel Electrophoresis

The amplified products were run through an electrophoresis procedure on 2% agarose gel (0.75 g of agarose in 50 ml of 1x TBE buffer - Appendix), stained with 5µl of ethidium bromide in the medium of 1x TBE buffer, and to allow the gel to solidify. It was kept at room temperature for 20 minutes[35]. Then after 6 µl of the PCR product were loaded into the wells to detect band size, amplicons were run simultaneously with a 3000-bp DNA ladder (South Korea). The gel was operated at 90 V of voltage for 45 minutes. After then, the gel was visualized using a UV trans-illuminator[36]. PCR fragments of the ITS region ranged from 550-570 bp, as shown in Figure 5[40, 39, 31].

2.7 DNA Sequence and Phylogenetic Analysis

The DNA sequencing was done by (Macrogen Company, Seoul, South Korea) for at least 1 to 3 isolates of each *Fusarium* species depending on amplified ITS region using **ITS1** (3-5) Forward (TCCGTAGGTGAACCTGCG) and **ITS4** (3-5) Reverse (TCCTCCGCTTATTGATATGC) primers. The phylogenetic tree and all analyzes were performed using the MEGA program version 11.0.13.

2.8 Statistical Analysis

One sample T-test was performed using the SPSS software (version 22.0), and differences between *Fusarium* species were considered significant at $P < 0.05$ for all corn fields.

3. Results and Discussion

3.1 Morphological Identification of Fusarium species

Among 50 samples of corn where collected in 30 corn fields, 39 isolates of *Fusarium* were detected. Based on morphological characteristics, six species of *Fusarium* were identified, namely *F. verticillioides*, *F. proliferatum*, *F. oxysporum*, *F. incarnatum*, *F. equiseti* and *F. fujikuroi* as presented in table 2 and figures 4. The number of isolates according to corn fields was 5 in Hawler, 5 in Kirkuk, 10 in Prde, 7 in Dbs, 6 in Ranya, 4 in Chwarqurna and 2 in Qarabelax. At the same time, there were no isolates in Kalar, Bazean, Halabja, Said sadiq, Chamchamal, Sangaw and Sarqala. Between all *Fusarium* isolates, 33.34% of isolates belonged to *F. verticillioides* which was significantly ($P < 0.05$) higher than all other species, followed by *F. proliferatum* (25.64%), *F. oxysporum* (12.82%), *F. incarnatum* and *F. equiseti* (10.25% each), then *F. fujikuroi* (7.7%) as shown in table 3.

The current study represents the first attempt to detect the *Fusarium* species colonizing corn in the Kurdistan region of Iraq, where corn cultivation started to increase in recent years. Several researchers have reported the occurrence of main Fusarium species in corn fields, such as F. verticillioides, F. proliferatum,

and F. equiseti[2]. The same species were recorded in this study. Some *Fusarium* species, such as *F. verticillioides*, *F. proliferatum*, *F. oxysporum*, *F. bullatum* and *F. thapsinum* were recovered from maize ears collected from 11 different geographic regions in Iran during 2004 and 2005[30], while in Indonesia, a research study found four species of mycotoxigenic *Fusarium* species isolated from maize based on molecular identification, which were *Fusarium verticillioides*, *F. proliferatum*, *F. graminearum* and *F. asiaticum*[41]. This study found that the occurrence of *F. verticillioides* in corn fields in the Kurdistan region was most commonly compared to other isolated species; this finding is consistent with those studies carried out in other countries such as Iran, India, Indonesia[37,36,30], and not agree with the results reported by research carried out in Egypt, which *F. oxysporum* is the most common species[34].

Table 2: Morphological characteristics of isolated Fusarium species.

Species	Colony Colour		Microscopic Features			
	Above	Revers	Macroconidia	Microconidia	Chlamydospore	Conidiophore
F. verticillioides	White pigmented violet	White violet	Slender to Ovoid, 3-5 Septa	Oval to club-shaped with a flattened base, 1-2septa on monophialides, may occur in V-shaped pairs	Absent	Apical end of the monophialide
F. oxysporum	Icy pink	White to peach	Straight to Slightly Curved, 3 Septa	Oval to reniform on aerial mycelia, 0 septa,	Present - singly or in pairs	Short monophialides
F. proliferatum	White orange	White orange to pale	Slender to Relatively Straight, 3-5 Septa	Club shaped in a chain, 0 septa.	Absent	Monophialides and polyphialides
F. incarnatum	Yellow red Or Dark red violet	White violet	Apical curved and Tapering Foot, 3-5 Septa	Fusiform	Sporodochia	Monophialides on aerial conidiophores
F. fujikuroi	White	White	Slender to Relatively Straight, 3-4 Septa	V-shaped microconidial in chains or obovoid	Absent	Mono- and polyphialides
F. equiseti	White Orange	White Orange	Foot Shaped & Elongated Foot, 4-6 Septa	Oval	Sporodochia	Apical taper

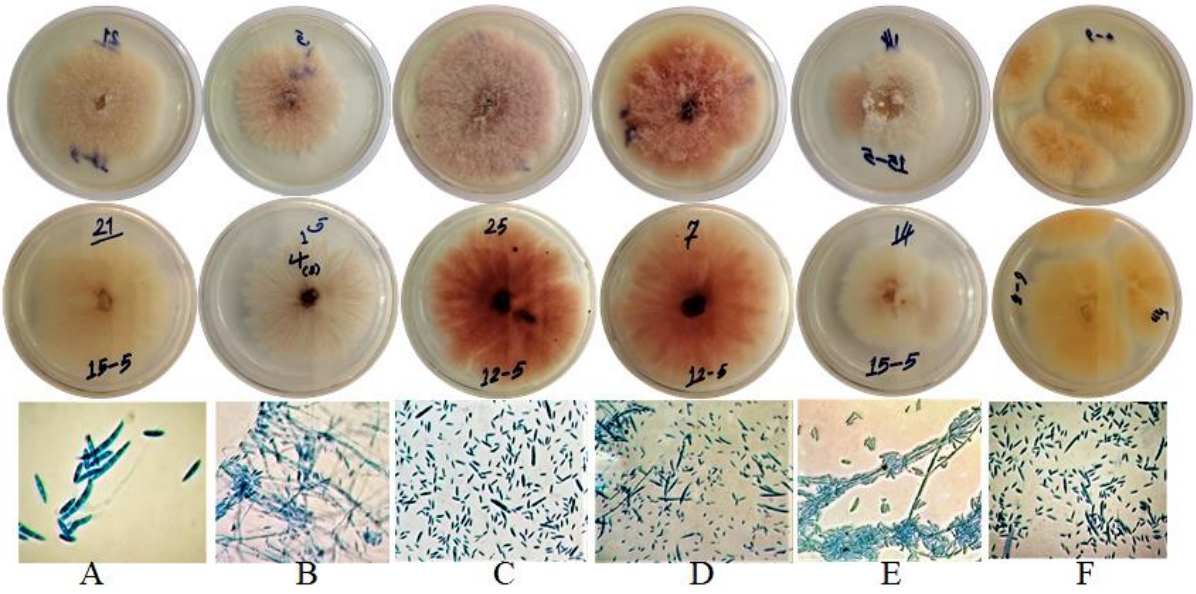

Figure 4: Morphological characteristics of different isolated *Fusarium* species: A (*F. verticillioides*), B (*F. oxysporum*) C (*F. proliferatum*), D (*F. incarnatum*), E (*F. fujikuroi*), F (*F. equiseti*).

Table 3: Isolated *Fusarium* species according to regions and their gene bank accession number.

Species and Accession Number	Hawler	Kirkuk	Prde	Dbs	Ranya	Chwarqurna	Qarabelax	Total
F. verticillioides (OQ421512)	2	2	2	3	1	1	2	13
F. oxysporum (OQ421511)	2	-	1	-	1	1	-	5
F. proliferatum (OQ408109)	-	-	4	2	3	1	-	10
F. incarnatum (OQ408107)	-	1	2	-	-	1	-	4
F. fujikuroi (OQ408106)	-	-	1	1	1	-	-	3
F. equiseti (OQ408111)	1	2	-	1	-	-	-	4
Total	5	5	10	7	6	4	2	39

3.2 Molecular Identification and DNA Sequence of Fusarium Species

All isolates were molecularly identified depending on ITS region to confirm the identification of *Fusarium* isolates by using forward ITS1 and reverse ITS4 primers. The expected 550-570 bp amplified ITS DNA product was detected in all 39 *Fusarium* isolates except in negative control, which confirms that all isolates belong to the *Fusarium* genus; Figure 5 represents the electrophoretic profile of the ITS region of *Fusarium* isolates. To ensure the *Fusarium* species identified by morphological characteristics, the PCR fragments of amplified ITS regions were sequenced, then aligned and registered in NCBI GenBank with the following accession numbers. *F. verticillioides* (OQ421512), *F. oxysporum* (OQ421511), *F. proliferatum* (OQ408109), *F. incarnatum* (OQ408107), *F. fujikuroi* (OQ408106) and *F. equiseti* (OQ408111) as shown in table 3. This study confirmed that PCR-based identification depending on conserved ITS region is highly accurate in differentiating the genus *Fusarium* from other fungal genera. This result is also reported in further research where done in North East India[43], Egypt[40] and Iran[39]. A PCR product of ITS region in *Fusarium* isolates from the current study ranged between 550-570bp; the same results are reported by other studies in Egypt[39,31] and in Iran[39].

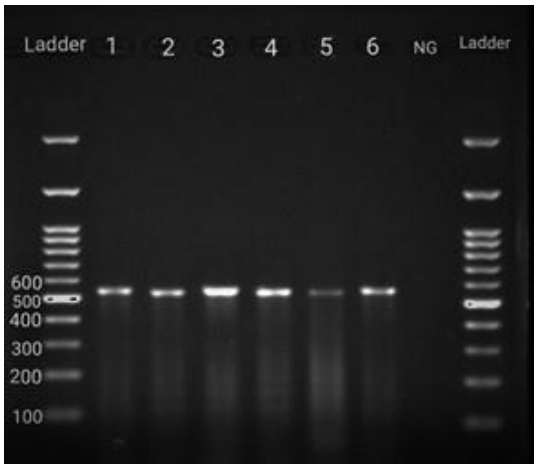

Figure 5: Agarose gel showing amplified products using primers for ITS region. NG (negative control), 1 (*F. proliferatum*), 2 (*F. verticillioides*), 3 (*F. equiseti*), 4 (*F. incarnatum*), 5 (*F. oxysporum*), 6 (*F. fujikuroi*).

3.3 Phylogenetic Analysis

The DNA sequences of ITS region using forward ITS1 and reverse ITS4 primers are used to generate a phylogenetic tree of *Fusarium* species, comparing to the other *Fusarium* strains in the public domain databases NCBI (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST). The evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model[44]. The tree with the highest log likelihood (-3089.56) is shown as the percentage of trees where the associated taxa clustered together is displayed next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 25 nucleotide sequences. There were a total of 478 positions in the final dataset. Evolutionary analyses were conducted in MEGA11[45]. As shown in Figure 6, all *Fusarium* species of the current study are divided into two main clusters; *F. fujikuroi*, *F. proliferatum* and *F. oxysporum* are clustered together on one side of the tree, while *F. verticillioides*, *F. equiseti* and *F. incarnatum* are located together in the other side. All details for comparing the *Fusarium* species of the current study to different *Fusarium* strains from other countries are determined on thephylogenetic tree.

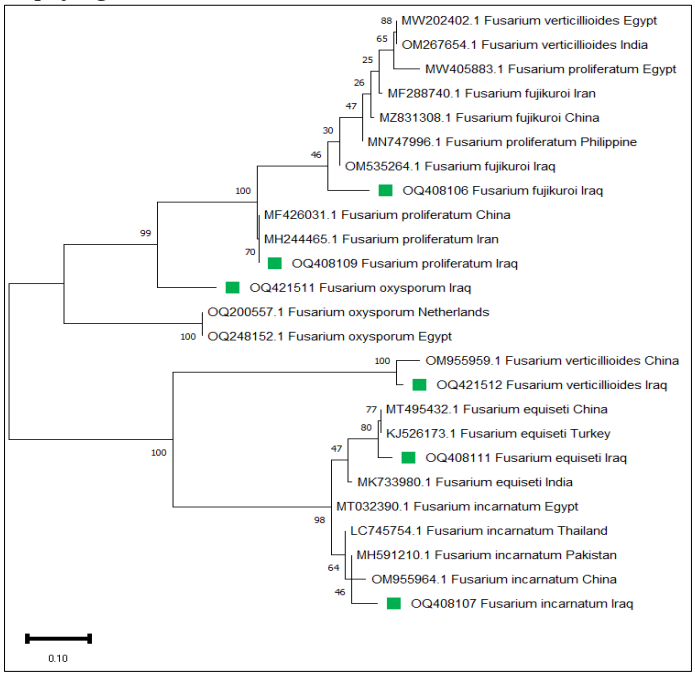

Figure 6: The phylogenetic tree generated using ITS region nucleotide sequence information of *Fusarium* species (The strains assigned with small green squares belong to the current study).

Conclusion

The current study concluded that the corn fields from seven locations in the Kurdistan region are infected with different *Fusarium* species, and the most common species is *F. verticillioides* as it is widely recovered among identified isolated. As well as, the *Fusarium* species in the Kurdistan region have close evolutionary history to the same species in other countries. Thus the current study recommends further investigations to detect the toxigenic *Fusarium* species involved in corn infections.

Conflict of interests

None

Author Contribution and Funding Information

Both authors fully contributed to the research, the first author mostly contributed to the practical part and writing, while the second author's contribution was supervision and revision. The research is partially funded by Charmo University and mostly is self-funded.

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