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MOLECULAR IDENTIFICATION OF ANGUINA FUNESTA THE CAUSAL AGENT OF BARLEY EAR COCKLE DISEASE IN NINEVEH PROVINCE – IRAQ

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Abstract

At the end of April 2020, a field survey was conducted in the barley fields of Nineveh province to detect barley Ear cockle disease caused by infection with nematode Anguina funesta and to estimate the percentages of infection with this disease, the percentage was 8,2,5,1,10% for each of Hamdaniya,Bashiqa, Bartella, Al-Boir and Khirbet Malak,. respectively. The infected samples were collected for several varieties of barley after obtaining the adult stages at the beginning of the formaation of the ears and comparing their morphological characteristics with those close up to them such as Anguina spp. Pathogenicity of the nematodes in barley plants was also confirmed by Koch's postulates. Molecular identification of Anguina isolates was done by amplifying the internal transcribed spacer (ITS) region of the conserved ribosomal DNA using primers ITS1 and ITS4. All the ITS sequences were compared for gaps and similarity sequences of the nematodes were homologous to those of A. funesta. isolates in the GenBank database with a similarity percentage of 95%, thereby confirming the identity of the causative agent of the disease. To the best of our knowledge this is the first record of A. funesta on the barley in Ninevah Governorate in Iraq. The nucleotide sequence of ITS from the Iraqi isolate has been assigned GenBank Accession No ON721332.1.

Keywords: Barley, Ear Cockle Disease, Anguina Funesta, PCR.

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Introduction

Excavations indicate that types of barley were cultivated 10,000 years ago in Iraq (Badr et al. 2000). At the present time, barley in Iraq is one of the main grain crops and is ranked second in importance after the wheat crop (Farhan et al. 2012). At the world level, it ranks fourth among the ten most important crops grown around the world (Akar et al. 2015).It is believed that the original home of barley is Ethiopia (Woldemichael 2019). The area planted with the barley crop in Iraq for the year 2020 amounted to 4.5 million dunums and nearly 3 million dunams for the year 2021. (Central Statistical Organization, Ministry of Planning). Also, barley is a major and important component of livestock and poultry feed (Gangwar et al. 2018). One of the most important pests that infect barley is the barley wart nematode *Anguina* sp. Where it was recorded for the first time in 1986 in Nineveh Governorate (Al.Talib et al. 1986) . Infection causes the loss of almost a third of the crop when the percentage of infection is 50%.(Al-Tai 2003). The barley cultivars approved by farmers in Nineveh Governorate varied in their susceptibility to infection with this type of nematode. (Al-Taie et al. 1999) indicated that the cultivars of barley are: wild barley, local black, Iba 99, ICSAD 68, ICSAD 176, and Arefat, and other cultivars showed a variation in their susceptibility to infection with this nematode. Nematode, as it was found that wild barley and local black were the most sensitive cultivars to infection, in contrast to the selected strain cultivar, where the cultivars were more resistant to the pathogen. This pest has a global spread, as it was first recorded in India on barley in 1979 by (Bahatti et al., 1979). It is also spread in West Asia and North Africa (Sikora, 1988), Pakistan (Maqbool et al. 1988), China and Eastern Europe (Tesic, 1969). Reports indicate its spread in the Russian Federation, Australia, New Zealand, Egypt, Brazil and the United States (Swarup and Sosa-moss, 1990). What increases the economic importance of this pest is its relationship with the bacteria that cause the disease of spike disease, or the so-called Tundu yellow spike rot (Bahatti et al. 1978). One of the most important species of the genus Anguina is the *funesta* because of its close relationship with the toxic bacteria *Rathayibacter toxicus* (Li et al. 2015) and (Riley and ophel 1992) . The *Lolium multiflorum* or the so-called ryegrass is one of the hostess of this type of nematode, and this herb is of economic importance as a source of fodder in the United States, where it ranked first in production at the level of forage crops in Oregon and accounted for 69% of the total production in the United States for crops Feed in 2010 with a value of 5.5 million dollars (Leamaster, 2011). Every year, cases of poisoning of livestock due to ingestion of ryegrass feed due to a type of poison called coryneoxin produced by *Rathayibacter toxicus* bacteria transmitted by *A. funesta* (Li et al. 2015),(Riley and ophel 1992) nematodes are recorded annually. Cases of poisoning due to ryegrass feed were recorded in South Africa in 1981 and in Japan in 1997. It is believed that the cause is the import of contaminated feed from Australia (Nogawa et al. 1997), (Schneider, 1981).Methods based on tracing differences in the sequence of nitrogenous bases in the ITS region are very useful in distinguishing between species of Anguina spp.(Li et al.2015).Powers *et al.* (2001) reported that the identification of the different species of the genus Anguina by PCR technique depends on matching the rRNA nitrogenous base sequences within the ITS region and the 5.8S segment. or on the ITS-5,8S-ITS2 region as described by Subbotin et al. (2004) . According to Powers et al. (2001), it is possible to distinguish between A. tritici and its close relatives such as *A. funesta* and Subanguina wevelli by PCR-RFLP technology, and depending on the ITS region and the restrictive enzymes, Alu, Hha and Hinf, in separating these species. 99 Nematode (Powers et al 2001) It is difficult to rely on morphological measurements to distinguish between species of the genus *Anguina*, and even the shape of the tail, which is more conical in the case of *funesta* compared to *agrostis* species, may not be sufficient for the diagnosis (Meng et al. 2012)

Material and methods

Field survey

At the end of April 2020, a field survey was conducted in the barley fields of Nineveh province to detect barley Ear cockle disease caused by infection with nematode *Anguina* spp and to estimate the percentages of infection with this disease,included Hamdaniya,Bashiqa, Bartella, Al-Boir and Khirbet Malak, respectively.

Five isolates (populations) of barley galls were collected, from the Ninevah governorate. These five different isolates were used as a source of *Angunia* spp for nematode DNA extraction.

DNA extraction

DNA were extracted from several 2nd-stage juveniles for each five isolate, using worm Lysis buffer (WLB), which consisted of 10 mM Tris pH 8.2, 50 mM KCl, 0.45% Tween 20, 2.5 mM $MgCl_2$,0.05% gelatine, and 60 µg/ml Proteinase K. Nematode juveniles were crushed on the clean slide with 10 µl WLB under binocular microscopes and then transferred to a new PCR tube on ice with an extra 10 µl of WLB. The samples were frozen at -80 °C for 10 minutes, and then samples were warmed up to room temperature, after which incubated in water bath at 60 °C for 1 h and followed by a 95 °C incubation for 10 min to completely lyse the cells, digest the proteins, and inactivate proteinase K. Subsequently, the tube was cooled on ice and centrifuged at 6,000 rpm for 30 sec. [16]. The supernatant material containing the DNA was gathered and stored at -20 °C or directly used for PCR.

G-spin dna extraction kit, intron biotechnology, cat.no. 17045

Two primers were used for the amplification of the ITS-rRNA gene, which were forward (5'-GTTTCCGTAGGTGAACCTGC- 3') and reverse (5'-ATATGCTTAAGTTCAGCGGGT - 3') for nematode isolates populations. The amplification was performed in 25 µl reactions containing 12.5 µl Red MyTaq™ (Mix Master Mix), 1µl of each primer, and the 2 µl of DNA template with 8.5 µl of a double distilled water to obtain a final volume of 25 µl. The conditions of the PCR reaction were 95 °C for 4 min, followed by 35 cycles of 95 °C for 40 sec, 59°C for 40 sec, 72°C for 1 min, and a final extension of 72 °C for 10 min [17].

Agarose gel electrophoresis of DNA

Electrophoresis has been done to determine DNA pieces after the process of extraction or to detect the result of the interaction of PCR during the presence of the standard DNA to distinguish the bundle size of the outcome of the interaction of PCR on the Agarose gel.

Prepare of the Agarose gel

According to Sambrook *et al* (16), the agarose gel has been made in 1.5% condensation by melting 1.5 g of agarose in 100 ml of previously made TBE buffer. Agarose has been heated to boil then left to cool down at (45-50ºC). The gel has been poured in the pour plate in which the plate of agarose support has been prepared after fixing the comb to make holes that would hold the samples. The gel has been poured gently not to make air bubbles and left 30 minutes to cool down. The comb has been removed gently of the solid agarose. The plate has been fixed to its stand in the Electrophoresis horizontal unit represented by the tank used in the Electrophoresis. The tank has been filled with TBE buffer in which it covers the gel surface.

Preparation of sample

3 µl of the processor loading buffer (Intron / Korea) has been mixed with 5 µl of the supposed DNA to be electrophoresis (loading dye), after the mixing process, the process of

loading is now to the holes of the gel. An Electric current of 7 v\c2 has been exposed for 1-2 h till the tincture has reached to the other side of the gel. The gel has been tested by a source of the UV with 336 nm after put the gel in pool contain on 3µl Red safe Nucleic acid staining solution and 500 ml from distilled water.

Detection of Gene *ITS* by Using PCR:

Detection of ***ITS*** gene was conducted by using primers for amplification. A fragment of ***ITS*** was amplified using a forward primer (***ITS1*** F: 5′- TCCGTAGGTGAACCTGCGG -3′) and a reverse primer (***ITS4*** R: 5′ TCCTCCGCTTATTGATATGC-3′) (Primers set supplied by IDT (Integrated DNA Technologies company, Canada.). The PCR amplification was performed in a total volume of 25µl containing 1.5µl DNA, 5 µl Taq PCR PreMix (Intron, Korea), 1µl of each primer (10 pmol) then distilled water was added into tube to a total volume of 25µl.The thermal cycling conditions were done as follows: Denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45s, 52°C for 1 min and 72 °C for 1min with final incubation at 72 °C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem). The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302nm) after red stain staining (Intron Korea).

Sequencing and Sequence Alignment

The PCR products were separated on a 2% agarose gel electrophoresis and visualized by exposure to ultra violate light (302 nm) after Red Stain staining. Sequencing of gene was performed by national instrumentation center for environmental management (nicem) online at (http://nicem. Snu .ac. kr/main/? En _skin=index.html), biotechnology lab, machine is DNA sequencer 3730XL, Applied Biosystem), Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www. Ncbi .nlm .nih .gov) and BioEdit program.

Results and dissection

Field survey

The results of a field survey conducted at the end of April 2020, in the barley fields of Nineveh province to detect barley Ear cockle disease caused by the nematode *Anguina funesta* .Incidence of barley gall varied in the surveyed fields and the percentages of infection with this disease, were 8,2,5,1,10% for each of Hamdaniya,Bashiqa, Bartella, Al-Boir and Khirbet Malak, respectively. The results of the field survey in the barley fields of Nineveh Governorate revealed the presence of barley gall nematodes, and the highest percentage was 10% in the fields of Khirbet Malak and Al-Kharar, followed by the Hamdaniya fields with 8% .

DNA sequencing of the Internal Transcribed Spacer (ITS) PCR amplified five band size 720 bp for each isolates of barley galls .Fig (1) showed the typical amplification products of PCR reactions with the two primers using 2 µl of template DNA of *Anguina* spp isolates

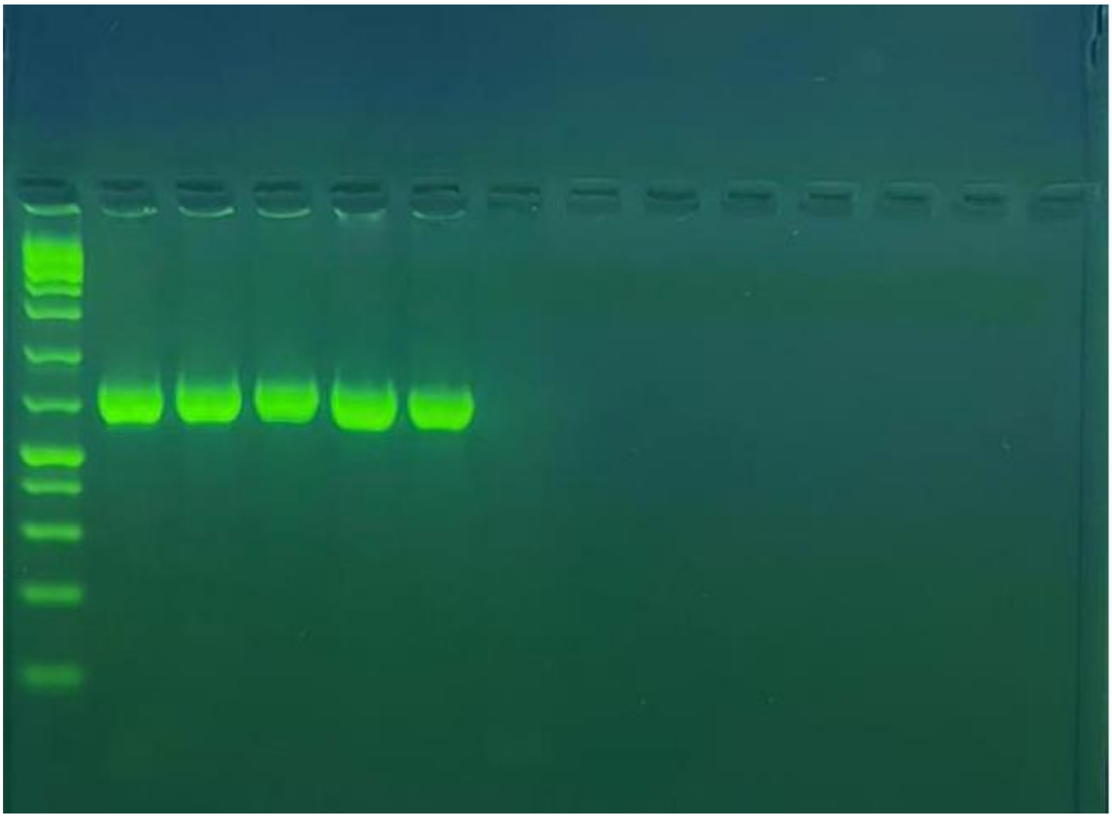

Figure (1) PCR product the band size 720 bp. The product was electrophoresis on 2% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (1000 plus)

Sequencing and Sequence Alignment

To confirm the morphological identification, the internal transcribed spacer (ITS) region of the *Anguina funesta* isolates were amplified with universal primers ITS1 and ITS4.The isolates were s partially diagnosed after conformity with the copies at the gene bank at National Center Biotechnology Information (NCBI) genes gave 96,97,96,95 and96% (diagnostic accuracy) match with isolation KM114435.1.The results obtained showed that different variations:, Transition have shown as showed Fig. 2,3,4,5and 6.

Score	Expec t	Identities	Gaps	Stran d
815 bits(441)	0.0	489/512(96%)	3/512 (0%)	Plus/ Plus

```
Query  1
ATGATGACTTCATTCTTACAGCCAATAGCCCAAGAAGGTGCCGTGATATTGGCATGCTGC  60
             ||||||| ||| |||||||||||||||| ||||||||||||| ||||| |||||
|||||
Sbjct  71
ATGATGAATTCGTTCTTACAGCCAATAGTCCAAGAAGGTGCCCTGATACTGGCACGCTGC  130

Query  61   TTCCAGGTGACGTCCCCACCGCTTAGCAGGCTTATTCTTGGGCG-
AAAAACGGCTTAGTT  119
             || | ||||  || ||||||||||||||||||||||||||||||
|||||||||||||||
Sbjct  131
TTACTGGTGCTGTGCCCACCGCTTAGCAGGCTTATTCTTGGGCGAAAAAACGGCTTAGTT  190
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Query  120
GGCTTCTAAGTTTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGCGTCGAAGAGAAAC  179
            ||||||||||||||||||||||||||| ||||||||||||||||||||| || |||| 
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Sbjct  191
GGCTTCTAAGTTTCTCTGAGCAGTTGTTTGCCTACGTCCGTGGCTGCGTTGAGGAGAGAC  250

Query  180
GGTACGTGGTCTTTGTGATCGCGAGAATCAATGAGTACCAGATAGGGTGCCGCCAACAAA  239
            ||||||||||||| 
||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  251
GGTACGTGGTCTTAGTGATCGCGAGAATCAATGAGTACCAGATAGGGTGCCGCCAACAAA  310

Query  240  ACAACCATTTTTGAATTTTTTGAGAA--
ATAACATTTCTAGTCTTACCGGTGGATCACTC  297
            ||||||||||||||| ||||||||||  ||||||||||||||||||
|||||||||||||
Sbjct  311
ACAACCATTTTTGAACTTTTTGAGAAACATAACATTTCTAGTCTTATCGGTGGATCACTC  370

Query  298
GGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGGTGTGAACTGCAGATATT  357

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Sbjct  371
GGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGGTGTGAACTGCAGATATT  430

Query  358
TTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATTTATCCTTTGGCACATCT  417

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  431
TTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATTTATCCTTTGGCACATCT  490

Query  418
GGCTCAGGGTCGTAAATACTAAACGAAAGCTATTCGTTGTTTATGACTGATTCATGGCTA  477
            |||||||||||||||||||||||||||||||||||||||||||||||
|||||||||||
Sbjct  491
GGCTCAGGGTCGTAAATACTAAACGAAAGCTATTCGTTGTTTATGACACATTCATGGCTA  550

Query  478  CACTAGTTAGGGCGATATTCCGCTAGAGCCATGTTTCTGTGAAG  521
            ||||||||||||||||| |||||||||| |||||||||||||||
Sbjct  551  CACTAGTTAGGGCGATACTCCGCTAGAGTCATGTTTCTGTGAAG  594
```

Figure 2. Anguina funesta isolate 1 : Sense flanking sequencing of partial *ITS* gene in comparison to gene standard of : KM114435.1, from Gene Bank. Sample query represents; Subject represent of database of (NCBI).

Score	Expect	Identities	Gaps	Strand
750 bits(406)	0.0	436/450(97%)	3/450 (0%)	Plus/ Plus

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Query  2    TGATATTGGCATGCTGCTTCCAGGTGACGTCCCCACCGCTTAGCAGGCTTATTCTTGGGC  61
            ||||| ||||| ||||||| | ||||  || |||||||||||||||||||||||||||||
Sbjct  114  TGATACTGGCACGCTGCTTACTGGTGCTGTGCCCACCGCTTAGCAGGCTTATTCTTGGGC  173

Query  62   G-AAAAACGGCTTAGTTGGCTTCTAAGTTTCTCTGAGCAGTTGTATGCCTACGTCCGTGG  120
            | |||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  174  GAAAAAACGGCTTAGTTGGCTTCTAAGTTTCTCTGAGCAGTTGTTTGCCTACGTCCGTGG  233

Query  121  CTGCGTCGAAGAGAAACGGTACGTGGTCTTTGTGATCGCGAGAATCAATGAGTACCAGAT  180
            |||||| || |||| ||||||||||||||| |||||||||||||||||||||||||||||
Sbjct  234  CTGCGTTGAGGAGAGACGGTACGTGGTCTTAGTGATCGCGAGAATCAATGAGTACCAGAT  293

Query  181  AGGGTGCCGCCAACAAAACAACCATTTTTGAATTTTTTGAGAA--ATAACATTTCTAGTC  238
            |||||||||||||||||||||||||||||||| ||||||||||  |||||||||||||||
Sbjct  294  AGGGTGCCGCCAACAAAACAACCATTTTTGAACTTTTTGAGAAACATAACATTTCTAGTC  353

Query  239  TTACCGGTGGATCACTCGGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGG  298
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  TTATCGGTGGATCACTCGGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGG  413

Query  299  TGTGAACTGCAGATATTTTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATT  358
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  TGTGAACTGCAGATATTTTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATT  473

Query  359  TATCCTTTGGCACATCTGGCTCAGGGTCGTAAATACTAAACGAAAGCTATTCGTTGTTTA  418
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  474  TATCCTTTGGCACATCTGGCTCAGGGTCGTAAATACTAAACGAAAGCTATTCGTTGTTTA  533

Query  419  TGA  421
            |||
Sbjct  534  TGA  536
```

Figure 3. Anguina funesta isolate 2 Sense flanking sequencing of partial *ITS* gene in comparison to gene standard of : KM114435.1, from Gene Bank. Sample query represents; Subject represent of database of (NCBI).

Score	**Expect**	**Identities**	**Gaps**	**Strand**
815 bits(441)	0.0	489/512(96%)	3/512 (0%)	Plus/ Plus

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Query  1
TTCTTACAGCCAATAGCCCAAGAAGGTGCCGTGATATTGGCATGCTGCTTCCAGGTGACG   60
            |||||||||||||||| ||||||||||||| ||||| ||||| ||||||| | ||||
|
Sbjct  83
TTCTTACAGCCAATAGTCCAAGAAGGTGCCCTGATACTGGCACGCTGCTTACTGGTGCTG   142

Query  61    TCCCCACCGCTTAGCAGGCTTATTCTTGGGCG-
AAAAACGGCTTAGTTGGCTTCTAAGTT   119
            | ||||||||||||||||||||||||||||||
|||||||||||||||||||||||||||
Sbjct  143
TGCCCACCGCTTAGCAGGCTTATTCTTGGGCGAAAAAACGGCTTAGTTGGCTTCTAAGTT   202

Query  120
TCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGCGTCGAAGAGAAACGGTACGTGGTCT   179
            ||||||||||||||| ||||||||||||||||||||| || ||||
||||||||||||||
Sbjct  203
TCTCTGAGCAGTTGTTTGCCTACGTCCGTGGCTGCGTTGAGGAGAGACGGTACGTGGTCT   262

Query  180
TTGTGATCGCGAGAATCAATGAGTACCAGATAGGGTGCCGCCAACAAAACAACCATTTTT   239
            |
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Sbjct  263
TAGTGATCGCGAGAATCAATGAGTACCAGATAGGGTGCCGCCAACAAAACAACCATTTTT   322

Query  240   GAATTTTTTGAGAA--
ATAACATTTCTAGTCTTACCGGTGGATCACTCGGTTCATAGATC   297
            ||| ||||||||||  ||||||||||||||||||
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Sbjct  323
GAACTTTTTGAGAAACATAACATTTCTAGTCTTATCGGTGGATCACTCGGTTCATAGATC   382

Query  298
GATGAAGAACGCAGCCAACTGCGATATATGGTGTGAACTGCAGATATTTTGAACACCAAG   357

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Sbjct  383
GATGAAGAACGCAGCCAACTGCGATATATGGTGTGAACTGCAGATATTTTGAACACCAAG   442

Query  358
AATTCGAATGCACATTGCGCCACTGGATATTTATCCTTTGGCACATCTGGCTCAGGGTCG   417

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Sbjct  443
AATTCGAATGCACATTGCGCCACTGGATATTTATCCTTTGGCACATCTGGCTCAGGGTCG   502

Query  418
TAAATACTAAACGAAAGCTATTCGTTGTTTATGACTGATTCATGGCTACACTAGTTAGGG   477
            |||||||||||||||||||||||||||||||||||
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Sbjct  503
TAAATACTAAACGAAAGCTATTCGTTGTTTATGACACATTCATGGCTACACTAGTTAGGG  562

Query  478  CGATATTCCGCTAGAGCCATGTTTCTGTGAAG  509
            ||||| |||||||||| |||||||||||||||
Sbjct  563  CGATACTCCGCTAGAGTCATGTTTCTGTGAAG  594
```

Figure 4. Anguina funesta isolate 3 Sense flanking sequencing of partial *ITS* gene in comparison to gene standard of : KM114435.1, from Gene Bank. Sample query represents; Subject represent of database of (NCBI).

Score	Expect	Identities	Gaps	Strand
826 bits(447)	0.0	499/524(95%)	3/524 (0%)	Plus/ Plus

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Query  1
ATGATGACTTCATTCTTACAGCCAATAGCCCAAGAAGGTGCCGTGATATTGGCATGCTGC  60
              ||||||| ||| |||||||||||||||| ||||||||||||| |||||| ||||
|||||
Sbjct  71
ATGATGAATTCGTTCTTACAGCCAATAGTCCAAGAAGGTGCCCTGATACTGGCACGCTGC  130

Query  61   TTCCAGGTGACGTCCCCACCGCTTAGCAGGCTTATTCTTGGGCG-
AAAAACGGCTTAGTT  119
              || | ||||  || ||||||||||||||||||||||||||||||
|||||||||||||||
Sbjct  131
TTACTGGTGCTGTGCCCACCGCTTAGCAGGCTTATTCTTGGGCGAAAAAACGGCTTAGTT  190

Query  120
GGCTTCTAAGTTTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGCGTCGAAGAGAAAC  179
              ||||||||||||||||||||||||||| ||||||||||||||||||||| || |||
||
Sbjct  191
GGCTTCTAAGTTTCTCTGAGCAGTTGTTTGCCTACGTCCGTGGCTGCGTTGAGGAGAGAC  250

Query  180
GGTACGTGGTCTTTGTGATCGCGAGAATCAATGAGTACCAGATAGGGTGCCGCCAACAAA  239
              |||||||||||||
||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  251
GGTACGTGGTCTTAGTGATCGCGAGAATCAATGAGTACCAGATAGGGTGCCGCCAACAAA  310

Query  240  ACAACCATTTTTGAATTTTTTGAGAA--
ATAACATTTCTAGTCTTACCGGTGGATCACTC  297
              ||||||||||||||| ||||||||||  ||||||||||||||||||
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Sbjct  311
ACAACCATTTTTGAACTTTTTGAGAAACATAACATTTCTAGTCTTATCGGTGGATCACTC  370

Query  298
GGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGGTGTGAACTGCAGATATT  357

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Sbjct  371
GGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGGTGTGAACTGCAGATATT  430

Query  358
TTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATTTATCCTTTGGCACATCT  417

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Sbjct  431
TTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATTTATCCTTTGGCACATCT  490

Query  418
GGCTCAGGGTCGTAAATACTAAACGAAAGCTATTCGTTGTTTATGACTGATTCATGGCTA  477
              |||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  491
GGCTCAGGGTCGTAAATACTAAACGAAAGCTATTCGTTGTTTATGACACATTCATGGCTA  550

Query  478  CACTAGTTAGGGCGATATTCCGCTAGAGCCATGTTTCTGTGAAG  521
            ||||||||||||||||| |||||||||| |||||||||||||||
Sbjct  551  CACTAGTTAGGGCGATACTCCGCTAGAGTCATGTTTCTGTGAAG  594
```

Figure 5 Anguina funesta isolate 4 Sense flanking sequencing of partial *ITS* gene in comparison to gene standard of : KM114435.1, from Gene Bank. Sample query represents; Subject represent of database of (NCBI).

Score	**Expect**	**Identities**	**Gaps**	**Strand**
684 bits(370)	0.0	406/423(96%)	3/423 (0%)	Plus/ Plus

```
Query  2    TGATATTGGCATGCTGCTTCCAGGTGACGTCCCCACCGCTTAGCAGGCTTATTCTTGGGC  61
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Sbjct  114  TGATACTGGCACGCTGCTTACTGGTGCTGTGCCCACCGCTTAGCAGGCTTATTCTTGGGC  173

Query  62   G-AAAAACGGCTTAGTTGGCTTCTAAGTTTCTCTGAGCAGTTGTATGCCTACGTCCGTGG  120
            | |||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  174  GAAAAAACGGCTTAGTTGGCTTCTAAGTTTCTCTGAGCAGTTGTTTGCCTACGTCCGTGG  233

Query  121  CTGCGTCGAAGAGAAACGGTACGTGGTCTTTGTGATCGCGAGAATCAATGAGTACCAGAT  180
            |||||| || |||| ||||||||||||||| |||||||||||||||||||||||||||||
Sbjct  234  CTGCGTTGAGGAGAGACGGTACGTGGTCTTAGTGATCGCGAGAATCAATGAGTACCAGAT  293

Query  181  AGGGTGCCGCCAACAAAACAACCATTTTTGAATTTTTTGAGAA--ATAACATTTCTAGTC  238
            |||||||||||||||||||||||||||||||| ||||||||||  |||||||||||||||
Sbjct  294  AGGGTGCCGCCAACAAAACAACCATTTTTGAACTTTTTGAGAAACATAACATTTCTAGTC  353

Query  239  TTACCGGTGGATCACTCGGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGG  298
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  TTATCGGTGGATCACTCGGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGG  413

Query  299  TGTGAACTGCAGATATTTTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATT  358
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  TGTGAACTGCAGATATTTTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATT  473

Query  359  TATCCTTTGGCACATCTGGCTCAGGGTCGTAAATACTAAACGAAAGCTATTCGTTGTTTA  418
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  474  TATCCTTTGGCACATCTGGCTCAGGGTCGTAAATACTAAACGAAAGCTATTCGTTGTTTA  533

Query  419  TGA  421
            |||
Sbjct  534  TGA  536
```

Figure 6. Anguina funesta isolate 5 Sense flanking sequencing of partial ITS gene in comparison to gene standard of : KM114435.1, from Gene Bank. Sample query represents; Subject represent of database of (NCBI).

Results showed the close genetic relationship among *A.funesta* isolated in this study (indicated with black prism) and those worldwide deposited in genbank database. Figure (7) represents comparison between local Iraqi isolate strain of *A.funesta* with the 6 strains of *A.funesta* recorded in the National Center Biotechnolgy Information (NCBI) and isolated from different countries showed compatibility 89% with *A.funesta* from USA . The nucleotide sequence of ITS from the Iraqi isolate has been assigned GenBank Accession No **ON** 721332.1. Thus, morphological and molecular examination confirmed the species as *A. funesta* . This is a first record of *A. funesta* in Ninevah governorate North Iraq and molecularly confirmed record of this species on barley .

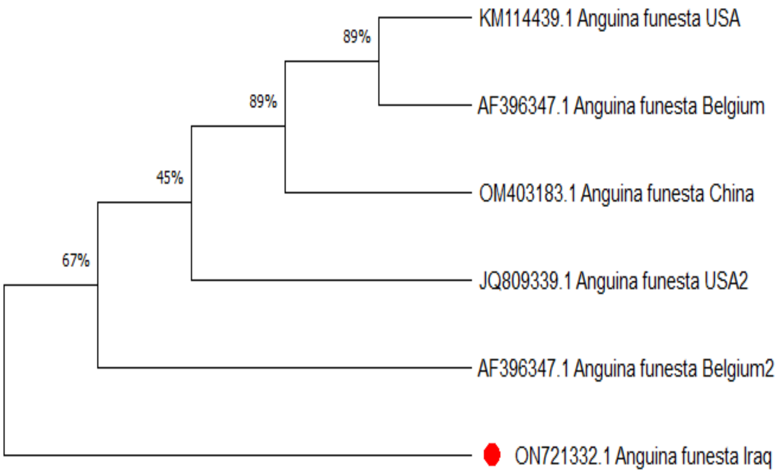

Figure (7) Phylogenetic tree constructed by the neighbor-joining method showing the phylogenetic relationships of *Anguina funesta* compared with the reference sequences from gene bank.

References

Akar,T., A., Muzaffer and F. Dusunceli. 2004. Barley post-harvest technologies . http:www.fao.org/inpho/content/compend/text/ch31/h31.htm. Accessed: August,2015.p64.

Al-Taae, A. K. (2003). Estimation of losses resulting from infection with barley gall and the effect of planting dates on reducing the incidence of the disease. Iraqi Journal of Agricultural Sciences 34:125-128

Al-Taae, A. K., Ali H. A., and Al-Taleb N.Y. (1999). Survey and selection of a strain of barley resistant to the barley gall nematode Anguina tritici in Nineveh Governorate. Mesopotamia Journal of Agriculture, 31, (2) :97-102

Al-Talib,N.Y., A.K.M. Al-Taae. S.M. Namer. Z.A. Stephan and A.S. Al-baldawi.(1986). New record of *Anguina tritici* on barley from Iraq. International Nematology Network Newsletter. 3:25-27.

Badr, A. Muller, K.Schafer-pergl, R.El-Rabey,H.Effgen, Ibrahim, H.H. Pozzi, C., Rohde,W. and Salamini, F. 2000. On the origin and Domestication History of Barley *(Hordeum vulgare*) Molecular Biology and Evolutionary Vol. 17. P.499-510.

Bhatti,D.S., Dahiya,R.S. and Dhawan,S.C. (1978). New record of tundu and earcockle in barley,Nematolgica 24,331-332.

Bhatti,D.S., R.S. Dahiya and S.C. Dhawan.(1978). New record of tundu and earcockle incidence in barley . Nematologica.24:331-332.

Edgar,J.A., Fralm, J.L. Corkrum, P.A., Anderson, N.,Jago, M.V., Culvenor,C.C.J., Jones, A.J., Murray, K., and Shaw, K.J. 1982. Corynetoxins causative agents of annual ryegrass toxicity :Their identification as tunicamycin group antibiotecs .J. Chem.Commun. 4:222-224.

Galloway, J.H.,1961. Grass seed nematode poisoning in livestock. J.AM. Vet. Med. Assoc.139:1212-1214.

Gangwar,O., Prasad, P., Bhardwaj, S.C., and Kumar, S., Barley diseases and their managment an Indian perspective, Reseach gate December 2018.

Jeanmougin,F.,Thompson, D.J.,Gouy, M., Higgins, D.G., and Gibson,T.J. 1998. Multiple sequence alignment with Clustal X. Trends Biochem. Sci.23:403-405.

Leamaster,K.K.,ed.2011. Oregon Agripedia. Vol.5. Oregon Department of Agriculture. Salem. OR.

Li,W., Zonghe, Y., M.K., and Skantar, A.M., 2015. Real-time PCR methods for detection and identification of the nematodes *Anguina funesta*, *A.agrostis, A.tritici*, and *A. pacificae*. Plant Dis. 99:1584-1589.

Maqbool, A.M.(1986). Classification and Distribution of plant parasitic Nematodes in Pakistan . National Nematological Research Center uneversity of Karatchi, Pakistan. 58pp.

Meng, S., Aldeman, S., Fraley, C., Ludy,R., Sun,F., and Osterbauer,N. 2012. Identification of *Anguina funesta* from annual ryegrass seed lots in oregon . online . Plant Health Progress dio:10.1094/PHP-2012-1024-01-RS.

Nogawa, M., Ishikawa, T., Miyazaki, S., Sato, W.,Taneichi, A., and Kobayashi, M.1997. Ryegrass intoxication in cattle and sheep fed oaten hay imported from Australia. J. Jpn Vet. Med. Assoc. 50:321-326.

Powers,T.O., Szalanski, A.L., Mullin, P.G., Harris, T.S., Bertozzi, T. and Griesbach, j.a. 2001. Identification of seed gall nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. Journal of Nematology . 33:191-194.

Price, P.C., Fisher, J.M., and Kerr, A. 1979. On *Anguina funesta* n. Sp. and its association with *Corynebacterium* sp. in infecting *Lolium rigidum*, Nematologica,25:76-85.

resistant Barley variety to the ear cockle nematode *Anguina tritici* in Nineveh Governorate. Rafidane Journal of Agriculture, 31(2):97 - 102

Riley,I.T., and Ophel, K.M.,1992. Clavibacter toxicus sp. nov.,the bacterium responsible for annual ryegrass toxicity in Australia. Int. J.syst. Bacteriol. 42:64-68.

Schneider,D.J. 1981. First reportfrom annual ryegrass toxicity in the Republic of south Africa. Onderstepoort J. Vet. Res.48:251-255.

Sikora, R.A.,1988. Plant parasitic nematodes of wheat and barley in temerature and temperate semi-arid regions - a comparative analysis. In M.C. Saxena, R.A. Sikora and J.P. Srivastava, eds . Nematodes parasitic to cereals and legumes in temperate semi-arid regions, p. 46-48. Aleppo, Syria, ICARDA.

Swarup, G. and Sosa-moss, C. 1990. Nematodes parasites of cereals. In M. Luc, R.A. Sikora and J. Bridge. eds. Plant parasitic nematodes in subtropical and tropical agriculture, p. 109-136 Wallingford, UK, CAB International.

Tesic, T.1969. A study on the resistance of wheat varieties to wheat eelworm*(Anguina tritici* Stein) . *Savrenema poljoprivreda,*17:541-543.

Woldemichael, M.D., (2019). Importance, Biology, Epidemiology,and managment of loose smut (*Ustilago nuda)* of Barley *(Hurdeum Vulgare*) East African Jornal of science, Vol 13. No 1 .

Yu, L.Z., Song, S.Y., Yu, C., Jiao,B.B. Tian,Y.M. and Li,Y.J. 2020. Rapid detection of *Anguina agrostis* by loop-mediated isothermal amplification. European Journal of Plant pathalogy 156:819-825.