

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 formation 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 (Tescic, 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 corynoxin 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 MgCl₂, 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), 1 µl 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 (45-50°C). 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/cm 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, 92°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 violet 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](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


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Query 120
GGCTTCTAAGTTTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGCGTCGAAGAGAAAC 179
      |||
Sbjct 191
GGCTTCTAAGTTTCTCTGAGCAGTTGTTTGCCTACGTCCGTGGCTGCGTTGAGGAGAGAC 250

Query 180
GGTACGTGGTCTTTGTGATCGCGAGAATCAATGAGTACCAGATAGGGTGCCGCCAACAAA 239
      |||
Sbjct 251
GGTACGTGGTCTTAGTGATCGCGAGAATCAATGAGTACCAGATAGGGTGCCGCCAACAAA 310

Query 240 ACAACCATTTTGGAATTTTTTGGAGAA--
ATAACATTTCTAGTCTTACCGGTGGATCACTC 297
      |||
Sbjct 311
ACAACCATTTTGGAACTTTTTGGAGAAACATAACATTTCTAGTCTTATCGGTGGATCACTC 370

Query 298
GGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGGTGTGAACTGCAGATATT 357
      |||
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).


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Sbjct 503  
TAAATACTAAACGAAAGCTATTCGTTGTTTATGACACATTCATGGCTACACTAGTTAGGG 562  
  
Query 478 CGATA TTCGCTAGAGCCATGTTTCTGTGAAG 509  
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||  
Sbjct 563 CGATA CTCGCTAGAGTCATGTTTCTGTGAAG 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 |

```

Query 1
ATGATGACCTTCATTCTTACAGCCAATAGCCCAAAGAAGGTGCCGTGATATTGGCATTGCTGC 60
      |||||  |||  |||||||||||||||  |||||||||||  ||||  |||||
|||||
Sbjct 71
ATGATGAAATTCGTTCTTACAGCCAATAGTCCAAAGAAGGTGCCCTGATACTGGCACCGCTGC 130

Query 61  TTCCAGGTGAACGTCCCACCGCTTAGCAGGCTTATTCTTGGGCG-
AAAAACGGCTTAGTT 119
      ||  ||||  ||  |||||||||||||||  |||||||||||
|||||||||||||||
Sbjct 131
TTACTGGTGTCTGTGCCACCGCTTAGCAGGCTTATTCTTGGGCGAAAAAACGGCTTAGTT 190

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

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

Query 240  ACAACCATTTTTGAATTTTTTGAGAA--
ATAACATTTCTAGTCTTACGGTGGATCACTC 297
      ||||||||||||  ||||||||  |||||||||||
|||||||||||
Sbjct 311
ACAACCATTTTTGAACTTTTTTGAGAAACATAACATTTCTAGTCTTATCGGTGGATCACTC 370

Query 298
GGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGGTGTGAACTGCAGATATT 357
      |||||||||||||||||||  |||||||||||
Sbjct 371
GGTTCATAGATCGATGAAGAACGCAGCCAACTGCGATATATGGTGTGAACTGCAGATATT 430

Query 358
TTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATTTATCCTTTGGCACATCT 417
      |||||||||||||||||||  |||||||||||
Sbjct 431
TTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATTTATCCTTTGGCACATCT 490

Query 418
GGCTCAGGGTCGTAAACTTAAACGAAAGCTATTCGTTGTTTATGACTGATTCATGGCTA 477
      |||||||||||
|||||||||||

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| Score | Expect | Identities | Gaps | Strand |
|---------------|---|--------------|------------|---------------|
| ٦٨٤ bits(٣٧٠) | 0.0 | 4٠٦/٤٢٣(9٦%) | 3/٤٢٣ (0%) | Plus/ Plus |
| Query 2 | TGATA T TGGCA T GCTGCTT CC AGGT GAC GT CCC ACC GCT TAGCAGGCTTATTCTTGGGC | 61 | | |
| Sbjct 114 | TGATA C TGGCA C GCTGCTT ACT GGT GCT GT GCC ACC GCT TAGCAGGCTTATTCTTGGGC | 173 | | |
| Query 62 | G-AAAAACGGCTTAGTTGGCTTCTAAGTTTCTCTGAGCAGTTGT A TGCCTACGTCCGTGG | 120 | | |
| Sbjct 174 | GA AAAAACGGCTTAGTTGGCTTCTAAGTTTCTCTGAGCAGTTGT T TGCCTACGTCCGTGG | 233 | | |
| Query 121 | CTGCGT CGA AGAGA AA CGGTACGTGGTCTT T TGTGATCGCGAGAATCAATGAGTACCAGAT | 180 | | |
| Sbjct 234 | CTGCGT TGAG GAGA G ACGGTACGTGGTCTT AG TGATCGCGAGAATCAATGAGTACCAGAT | 293 | | |
| Query 181 | AGGGTGCCGCAACAAAACAACCATTTTTGAA T TTTTTGAGAA--ATAACATTTCTAGTC | 238 | | |
| Sbjct 294 | AGGGTGCCGCAACAAAACAACCATTTTTGAA C TTTTTGAGAA AC ATAACATTTCTAGTC | 353 | | |
| Query 239 | TTA C CGGTGGATCACTCGGTTCCATAGATCGATGAAGAACGCAGCCAAC T GCGATATATGG | 298 | | |
| Sbjct 354 | TTA T CGGTGGATCACTCGGTTCCATAGATCGATGAAGAACGCAGCCAAC T GCGATATATGG | 413 | | |
| Query 299 | TGTGAACTGCAGATATTTTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATT | 358 | | |
| Sbjct 414 | TGTGAACTGCAGATATTTTGAACACCAAGAATTCGAATGCACATTGCGCCACTGGATATT | 473 | | |
| Query 359 | TATCCTTTGGCACATCTGGCTCAGGGTCGTAATACTAAACGAAAGCTATTTCGTTGTTTA | 418 | | |
| Sbjct 474 | TATCCTTTGGCACATCTGGCTCAGGGTCGTAATACTAAACGAAAGCTATTTCGTTGTTTA | 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 Biotechnology 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 .

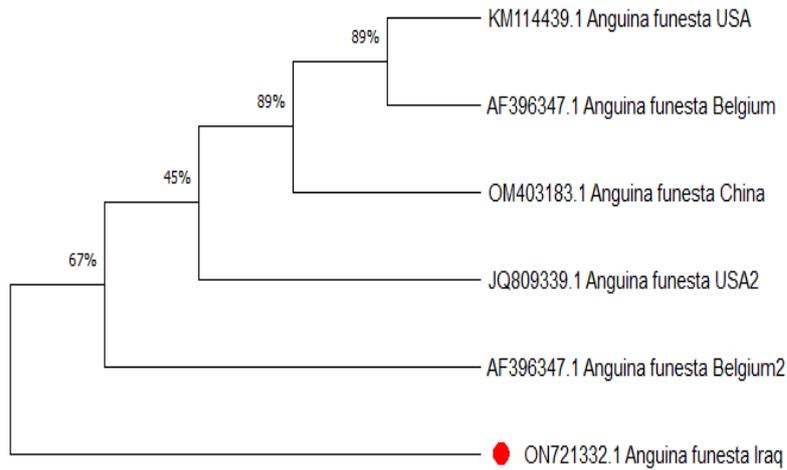


Figure (7) Phylogenetic tree constructed by the neighbor-joining method showing the phylogenetic relationships of *Anguina funesta* compared with the reference sequences from gene bank.

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