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TRICHODERMA LONGIBRACHIATUM: FIRST RECORD IN IRAQ

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Abstract
Trichoderma longibrachiatum is a soil fungus which often found on dead wood, other fungi, building material and sometimes animals. It is found all over the world but mainly in warmer climates. In this study, we isolated the fungal isolates from soils of fields of wheat in Iraq during 2017. The isolates was identified based on morphological characteristics by microscopic observation, determination of cultural features on different media and molecular phylogenetic analyses of internal transcribed spacer (ITS) rDNA. As a results, the isolate 48 namely Trichoderma longibrachiatum fungus was recorded as AB1 strain in National Center for Biotechnology Information (NCBI) for the first time in Iraq.

Keywords: First record, Trichoderma longibrachiatum fungi, phylogenetic analysis, ITS , NCBI, Iraq.

Introduction

Trichoderma sp. are widely distributed all over the world and occur in nearly all soils and other natural habitats, especially in those containing organic and inorganic mattar (1). They are ubiquitous colonizers of cellulosic materials and can thus often be found wherever decaying plant material is available as well as in the rhizosphere of plants, where they can induce systemic resistance against pathogens (2). They are filamentous fungi commonly found in the soil community that are facultative saprophytes. Thereafter, numerous new species of Trichoderma were discovered, and the genus already comprised more than 100 phylogenetically defined species (3).
Trichoderma longibrachiatum is a soil fungus which often found on dead wood, other fungi, building material and sometimes animals. It is found all over the world but mainly in warmer climates (4).

It was found a fast-growing fungus and it typically produces off-white colonies that change to greyish green with age (5). This species is able to grow over a wide range of temperature; however the optimal temperature for growth is ≥ 35 °C (6) .T. longibrachiatum is a clonal species that reproduces through 1-celled, smooth-walled conidia (7).

Therefore, the study was conducted to identify the Trichoderma fungal isolates from from soils of fields of wheat in Iraq. To our knowledge , this paper is reported for first finding in Iraq.

Materials and Methods

Collection of soil samples

Forty-eight soil samples were collected from fields cultivated with wheat crops from Abu- Ghraib/ Baghdad Governorate, Al- Najaf Governorate and Diyala Governorate during 2016 and assigned from N1 to N48. About 100 g portions of each soil sample was taken from a depth of about 15 cm from the soil surface and placed in a sterile polyethylene bag and transported to the laboratory of the college of biotechnology Al-Nahrain University.

Serial dilutions of each soil samples were prepared by mixing 1g with 9 ml of sterile distilled water, and shaked vigorously, then dilutions of 10-4 and 10-5 were carried out (8). Aliquots of 0.1ml of each dilution was spread on TSM agar medium as a selective medium for isolation of Trichoderma spp. (9), plates were then incubated at 28℃ for 7 days. After incubation, pure fungal isolates were subcultured on PDA agar plates as an inrichment medium for maintenance and identification of fungal isolates. Then plate were incubated at 28℃ for 7 days, and kept at 4℃ for further analysis.

Identification of Trichoderma spp

Morphological Identification

The soil samples were cultured on the TSM specific medium using serial dilution method that reached to dilution of 10-4 and 10-5 which were selected and incubated at 28°C for 7 days for growth of Trichoderma spp. only. After that, these fungal isolates were cultured on the special identification media for identification of the fungal isolates according to the genus (7). The identification media such SNA, CMA, CMD, as well as PDA, MEA which were used for dentification according to macroscopic and microscopic characteristics.

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The macroscopic characteristics of the colony (colony diameter, conidia color, presence of diffusible pigment, prescence of coconut odour, concentric rings and pustules) as well as the microscopic features (shape of conidiophores, phialides, conidia and chlamydospores) were used for identification to the species level using compound microscope. For microscopic characteristics slides were stained with Lactophenol cotton blue. Photographs were taken with Digital microscopical camera. Pure cultures of isolates were maintained in PDA slant medium with glycerol 25% and these were tightly wrapped with parafilm and placed at 4 °C in refrigerator as stock cultures and sub cultured was done every three months (10).

Molecular Identification of Trichoderma N48 isolate

Extraction of genomic DNA

Genomic DNA was extracted from the selected fungal isolate N48 using the kit maufacturer ZR Fungal DNA MiniPrep™ Kit supplied by Zymo Research Corporation company (11) as follows:

1. A 150 mg of fresh culture of fungal isolate from Trichoderma spp. N48 maintained on PDA agar medium at temperature 30 °C, 3 days and was suspended in 200 µl of deionized distilled water in a lysis tube and shaked vigorously.

2 .Aliquot of 750 µl of lysis solution was added, and mixed by vortexing for 5 minutes and centrifuged at 10000 rpm for 1 minute.

3 . A portion of 400 µl of supernatant was transferred into spin filter collection tube, and centrifuged at 8000 rpm for 1 minute.

4 .The filtarate was transferred to another collection tube, then 1.2 ml of fungal DNA binding buffer was added and mixed thoroughly.

5 .Aliquot of 800 µl of the mixture was added into a new spin colum in a collection tube and centrifuged at 10000 rpm for 1 minute.

6 .The flow through was discarded, then step 5 was repeated.

7 .A portion of 200 µl of DNA pre-wash buffer was added, mixed thoroughly, and centrifuged at 10000 rpm for 1 minute.

8 .The flow-through was discarded, then 500 µl of fungal DNA wash buffer was added into the new spin colum, mixed thoroughly and centrifuged at 10000 rpm for 1 minute.

9 .The flow-through was discarded.Then the DNA solution in the spin- colum was tranferred into a clean 1.5 ml microcentrifuge tube. After that 100 µl of DNA elution buffer was added and centrifuged at 10000 rpm for 30 second to elute the purified DNA.

Determination of DNA purity and concentration

Purity and concentration of DNA solutions were measured by using the nanodrop spectrophotometer by placing 2µl of DNA solution into the photocell of the apparatus, then optical density was measured at 260 nm and 280 nm.

Amplification of ITS regions of 5.8 S rRNA gene

PCR used to amplify the internal transcribed spacer regions 1 and 4 (ITS 1 and ITS 4) of rRNA gene cluster using the primer pair for amplification (table 1), these primers were supplied by IDT (Integrated DNA Technologies company, Canada).

Table (1): The PCR primers used for amplification of ITS regions of 5.8 S rRNA gene.

Primer name	Sequence(5′→3′)	T_m (°C)	GC content (%)	Product size(bp)	Reference
Forward ITS1	TCCGTAGGTGAACCTGCGG	60.3	50 %	650 (12)	White *et al.*, (1990)
Reverse ITS4	TCCTCCGCTTATTGATATGC	57.8	41 %		

The PCR amplification was performed in a total volume of 25µl in eppendrof tubes as following to the company's instructions (Intron/Korea) as shown in table (2). The total volume of PCR reaction was mixed by vortexing for 5 seconds and transferred to the thermal cycling (Multi Gene Optimax Gradient) using thermal cycling conditions were done as shown in (table 3).

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Table (2): The Components of the mixture PCR reaction conditions for the diagnosis of ITS regions of 5.8 S rRNA gene.

Components	Volume(µl)
Taq PCR PreMix	5
Forward primer (10 picomols/µl)	1
Reverse primer (10 picomols/µl)	1
DNA template	1.5
Distill water	16.5
Final volume	25

Table(3): The optimum PCR amplication conditions for amplification of ITS regions of 5.8 S rRNA gene (12).

No.	Phase	T_m (°C)	Time	No. of cycles
1	Initial Denaturation	94	3 min.	1 cycle
2	Denaturation	94	45 sec	35 cycle
3	Annealing	52	45sec	
4	Extension	72	45sec	
5	Final extension	72	10 min.	1 cycle

Gel electrophoresis for genomic DNA and PCR products

The gel electrophoresis was used to observe the genomic DNA and PCR products and it is prepared according to (13).

I. Prepared agarose gel electrophoresis

Powder agarose was added to 100 ml of 1X TBE buffer according to the desired concentration (1% to separated DNA and 2% separated PCR product) in a 200 ml flask. This was then gently heated in a microwave, with frequent mixing, until the agarose had dissolved. After allowing the gel to cool to 40 - 50 °C, red safe dye(5 µl) was added to the gel, the gel was mixed with red safe quietly. While allowing the gel to cool, a template was prepared (163mm×149mm) by inserting the casting unit in a casting holder and sealed.

A gel comb was inserted, the molten gel was then poured into the prepared unit, the gel comb was removed gently, and the gel casting unit containing the set gel was placed into the gel tank. The wells was put nearer the negative (black) electrode. 1X TBE buffer was then poured into the gel tank to fully submerge the gel.

II. DNA and PCR product agarose gel electrophoresis

To separate DNA fragments, agarose gels in different concentrations were used 1%for extracted DNA, 2% for visual checking of PCR product. Gels were run horizontally in 1 X TBE buffer. 5 µl of the DNA sample was loaded using 2 µl loading buffer, mixed well and added in to the wells also 5 µl of the PCR product was loaded in to the well. Electrophoresis was carried out for 1.5 hr at 70v at 5volt/cm in the prescence of a molecular weight marker 100 bp Kapa/ USA. After electrophoresis, the DNA bands were visualized using a UV transilluminator (Vilber lourmat / France) at 302 nm.

Sequencing of amplification products

Amplification products was sent for sequencing to Macrogen Company (Korean Biotechnology Company, Korea). Then, the sequencing for these products was compared with the information in gene bank of the National Center for Biotechnology Information (NCBI) for DNA sequencing and by using BLAST program of NCBI and Mega 6 program for phylogenetic analysis.

Results and Discussion

Identification of Trichoderma N48 isolate

Morphological characteristic

According to the taxonomic key of Trichoderma spp. of (7), this fungal isolate N48 was belong to the species Trichoderma longibrachiatum as shown in table (4) and figure (1), (2), (3), (4), (5), (6), (7), (8).

The macroscopic characteristics were observed on the media PDA and SNA whereas the microscopic characteristics were showed on the media CMA, CMD and MEA.

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Table(4): Characteristics of Trichoderma longibrachiatum

Characteristics	Observation
Colonies	The colony radius on PDA: 50-65mm SNA: 45-52mm • There are diffusing yellow pigment on PDA and no distinctive odor.
Condiation on PDA	Pustules abundant, uniformly dispersed sometimes in concentric rings, often forming a continuous lawn, becoming confluent, cottony or wooly, dark green; completely fertile conidiophores visible.
Branching pattern	Fertile branches typically consisting of several levels of branching, branches near the tip bearing a single phialide and not rebranched, branches distal to the tip longer and rebranching
Conidiophores	Complicated and progressively longer, often paired, secondary branches.
Phialids	Cylindrical to lageniform, swollen in, solitary, often terminating in a single cell
Condia	Ellipsoidal, green, smooth
Chlmydospores	Terminal and then subglobose to globose were observed on (CMD)
Growth	grows at 25°C but unable to grow at 5°C and 45° C.

Figure(1): Colonies on PDA of T.longibrachiatum after incubation for 7 days at 28°C.

Figure(2): Colonies on CMA of T. longibrachiatum after incubation for 7 days at 28°C.

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Figure(3): Colonies on CMD of T.longibrachiatum after incubation for 7 days at 28°C.

Figure(4): Colonies on MEA of T.longibrachiatum after incubation for 7 days at 28°C.

Figure (5): Colonies on SNA of T.longibrachiatum after incubation for 7 days at 28°C.

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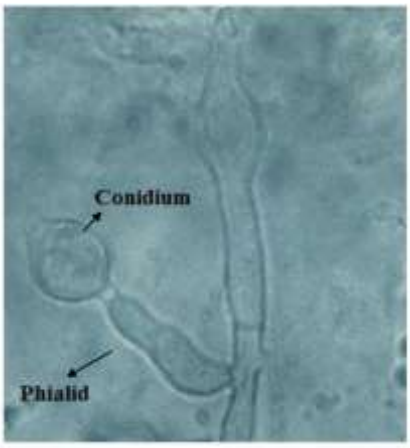
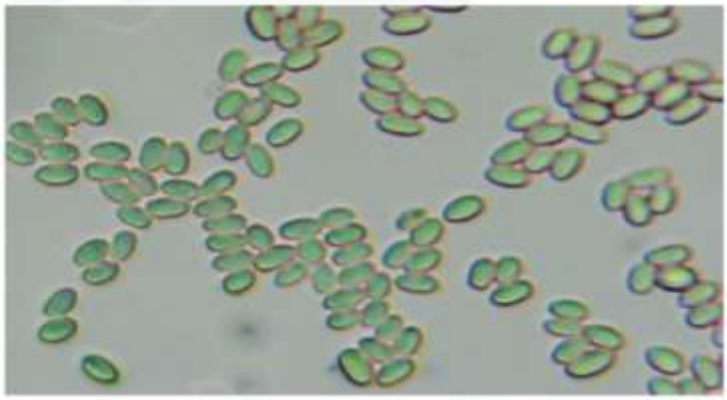


Figure (6): Phialides and Conidiophore on CMA under the microscope (100x).

Figure (7): Chlamydospores on CMD under the microscope (100x).



Figure(8): Conidia on SNA under the microscope (100x).

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Molecular identification

Extraction of genomic DNA

Genomic DNA of the T.longibrachiatum N48 was extracted in order to amplify ITS of 5.8s rRNA gene using polymerase chain reaction. Results of extraction using ZR Fungal/Bacterial DNA MiniPrep™Kit showed that DNA was extracted in a pure form according to the absorbance ratio (260/280) which was 1.8-2.0 with a concentration of 41.43 ng/µl. Results of electrophoresis on agarose gel (1%) are illustrated in figure (9) which show that only one DNA band with high resolution represents genomic DNA extracted from the wild type fungal isolate. Aliqoutes of this DNA were taken and used for amplification of ITS of 5.8s rRNAgene. The results agree with (14), who recorded that the DNA concentration of Trichoderma sp. ranged from 24.3- 117.4 ng/µl according to the level of species and purity ranging from 1.8-2.

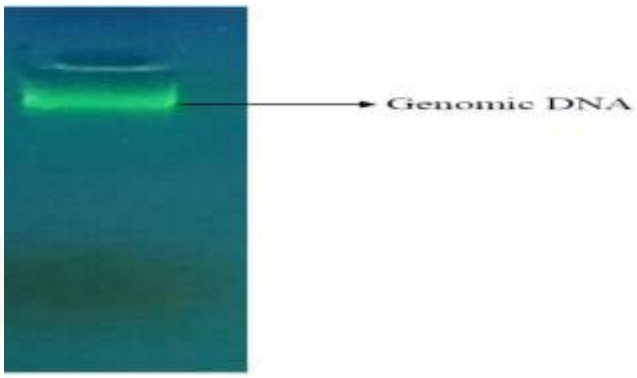

Figure (9): Genomic DNA of T.longibrachiatium N48 after electrophoresis on agarose gel (1%) at 5v/cm2, then exposed to UV light and photographed.

PCR amplification, sequencing and alignment

The genomic DNA was extracted from T.longibrachiatum and universal ITS -1 primers 19F and ITS–4 primers 20R were used for the amplification and sequencing of the ITS1,5.8rRNA gene, ITS2 region. In figure (10) a specific fragment of the expected size about 550bp of ITS1, 5.8rRNA gene, ITS2 region was sequenced and used for the identification of isolated fungal strain via blast search of genebank available in NCBI which resulted the sequence was deposited as accession number **KY750397.1** (figure 11) and the blast search has shown **100%** homology with the strain Trichoderma longibrachiatum as shown in table (5). After that, the resulting forward and reverse ITS sequences were alignmed using NCBI by blast search which was observed as 100% identifies of isolate (Figure 12). Finally, the sequencing of T. longibrachiatum isolate was then recorded in NCBI site as **AB1** for the first time in Iraq as shown in figure(13).

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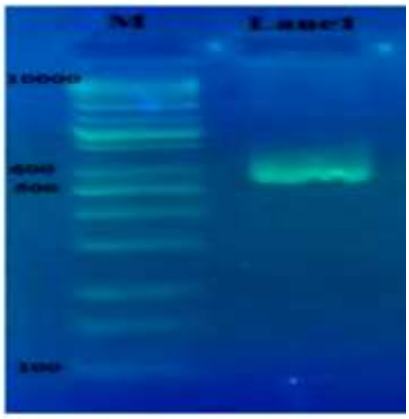

Figure (10): PCR products of ITS regions of 5.8S rRNA gene with a specific band size of 550bp. The products were electrophoresis on a 2% agarose gel at 5 volt/cm2. 1x TBE buffer for 1.5hrs. M: DNA ladder (100), lane1: T.longibrachiatum isolate .PCR products of band size 550 bp, visualized under U.V light.

```
1   cctggtgaag cggagggaca ttaccgagtt tacaactccc aaaccccaat gtgaacgtta
61  ccaatctgtt gcctcggcgg gattctcttg ccccgggcgc gtcgcagccc cggatcccat
121 ggcgcccgcc ggaggaccaa ctccaaactc ttttttctct ccgtcgcggc tcccgtcgcg
181 gctctgtttt atttttgctc tgagcctttc tcggcgaccc tagcgggcgt ctcgaaaatg
241 aatcaaaact ttcaacaacg gatctcttgg ttctggcatc gatgaagaac gcagcgaaat
301 gcgataagta atgtgaattg cagaattcag tgaatcatcg aatctttgaa cgcacattgc
361 gcccgccagt attctggcgg gcatgcctgt ccgagcgtca tttcaaccct cgaacccctc
421 cgggggggtcg gcgttggggg atcggccccc caccgggccg ccccccgaaat acagtggcgg
481 tctcgccgca gcctctcctg cgcagtagtt tgcacactcg caccgggagc gcggcgcggc
541 cacagccgta aaacacccca aacttctgaa atgttgacct cggatcaggt aggaataccc
601 gctgaactta agcatatcaa aaagccggag gaaa
```

Figure (11):Nucleotide Sequence of the Trichoderma longibrachiatum isolate using Sanger dideoxy sequencing internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence, genebank: KY750397.1.

No. of repeat	Type of substitution	Location	Nucleotide	Region	Sequence ID	Score	Expect	Identities	Source
1	None			74 to 473	KY750397.1	739	0.0	100%	*Trichoderma longibrachiatum*

Table(5):sequencing ID, Score, Expect and Identities of ITS regions of 5.8S rRNA gene of T. longibrachiatum N48.

Score	Expect	Identities	Gaps	Strand
739 bits(400)	0.0	400/400(100%)	0/400(0%)	Plus/Plus

```
Query  1    TCGGCGGGATTCTCTTGCCCCGGGCGCGTCGCAGCCCCGGATCCCATGGCGCCCGCCGGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  74   TCGGCGGGATTCTCTTGCCCCGGGCGCGTCGCAGCCCCGGATCCCATGGCGCCCGCCGGA 133

Query  61   GGACCAACTCCAAACTCTTTTTTCTCTCCGTCGCGGCTCCCGTCGCGGCTCTGTTTTATT 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  134  GGACCAACTCCAAACTCTTTTTTCTCTCCGTCGCGGCTCCCGTCGCGGCTCTGTTTTATT 193

Query  121  TTTGCTCTGAGCCTTTCTCGGCGACCCTAGCGGGCGTCTCGAAAATGAATCAAAACTTTC 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  194  TTTGCTCTGAGCCTTTCTCGGCGACCCTAGCGGGCGTCTCGAAAATGAATCAAAACTTTC 253

Query  181  AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  254  AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG 313

Query  241  TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATT 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  314  TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATT 373

Query  301  CTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGTCGGCG 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  374  CTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGTCGGCG 433

Query  361  TTGGGGGATCGGCCCCTCACCGGGCCGCCCCCGAAATACA  400
            ||||||||||||||||||||||||||||||||||||||||
Sbjct  434  TTGGGGGATCGGCCCCTCACCGGGCCGCCCCCGAAATACA  473
```

Figure (12): Sequence alignment of ITS regions of 5.8s RNA from Trichoderma longibrachiatum internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.Sequence ID: KY750397.1.

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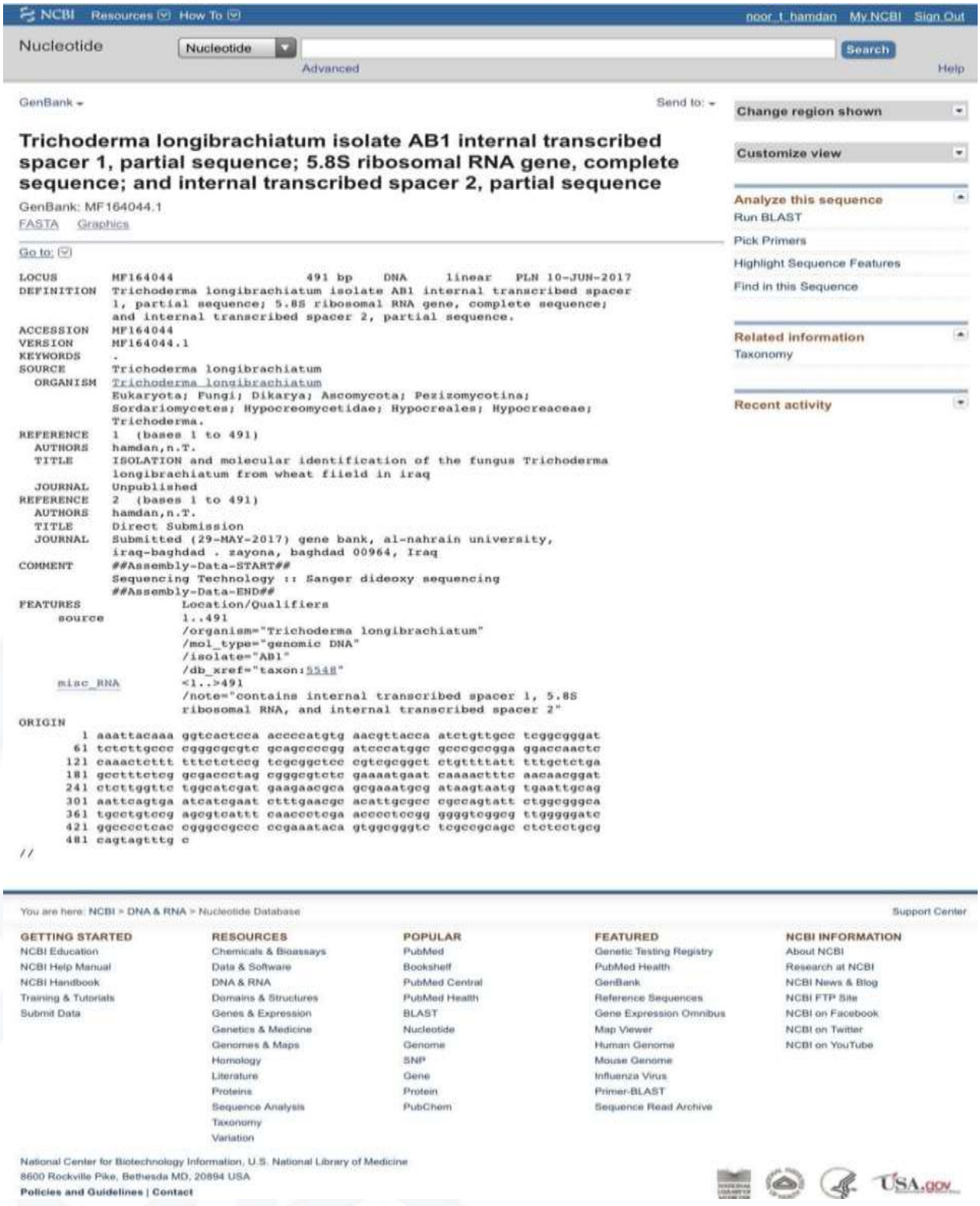

Figure(13): The isolate of T.longibrachiatum was recorded in NCBI site as AB1 https: //www.ncbi.nlm.nih.gov/nuccore/MF164044.1.

The amplification of 5.8S rRNA gene using primer pair ITS1 and ITS4 has been done. The two spacers, together with the 5.8S gene, are normally referred to as the ITS region (15). This is a very good tool of species identification because rRNA genes are universally conserved, highly stable while the ITS region is highly variable. The ITS region evolves very fast and even within a genus it may vary among species. Therefore, the sequences of these regions can be used for identification of closely related species (12).

The finding of this study in agreement with findings of (16) obtained a single product of approximately 560 to 600 bp from all the PCR amplifications with primers ITS1 and ITS4 for 17 biocontrol isolates of Trichoderma spp. Similarity with finding of (17) which referred that the PCR amplications with primers ITS1 and ITS4 for the biocontrol isolate of Trichoderma longibrachiatum was 600bp. ITS sequence analysis is a reliable method for phylogenetic analysis and species identification within the section longibrachiatum of the genus Trichoderma (18). (19) investigated the diversity of Trichoderma Poland utilizin a combination of morphological and molecular/phylogenetic methods, 170 Trichoderma isolates were identified to the species level by the analysis of their ITS1, ITS2 rDNA sequences.

Phylogenetic tree analysis

The neighbor-joining phylogenetic tree was constructed with these homologous sequences using MEGA v.6.0 software. The results showed that isolate N48 is a Trichoderma longibrachiatium. In figure (14), the phylogenetic relationships were deduced for 13 Trichoderma spp. sequences available in Genbank. The isolate N48 of Trichoderma longibrachiatum IRAQ was closely related at the nucleotide level to the Austria isolate (EU401554.1) T. longibrachiatum, Indian isolate (KY568698.1) T. longibrachiatum, USA isolate (KJ174236.1) T. longibrachiatum, Yunnan isolate (GQ203533.1) T. longibrachiatum, Israel isolate (FJ571489.1)T. longibrachiatum showed up to 100% of similarity, while in China isolate (KY764811.1) T. longibrachiatum, Poland isolate (KF889068.1) T. longibrachiatum, Saudi Arabia isolate (HE649491.1) T.longibrachiatum, Japan isolate (LC228634.1) T. longibrachiatum, Belarus: Minsk region isolate(KY346983.10) T. longibrachiatum, Indonesia isolate (LC185084.1) T. longibrachiatum, Italy isolate (KU935695.1) T. longibrachiatum, Egypt isolate (KU360155.1) T. longibrachiatum showed up to 99% of similarity as shown in figure (15) .

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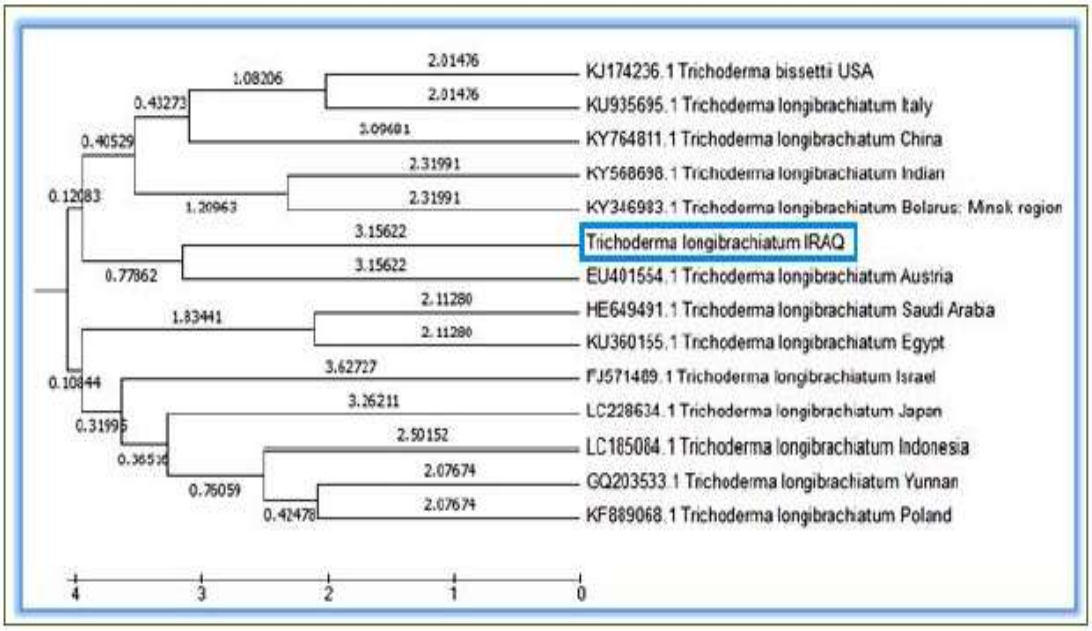

Figure(14): Phylogenetic tree analysis of T. longibrachiatum.

The domains of ITS and intergenic regions are much more variable and therefore more useful for phylogenetic studies of members of the same species or genus (12). The use of divergence sequences to define species requires careful interpretation, especially because length polymorphisms and inversions can make comparison on a simple basis difficult (20). Related to the phylogenetic tree obtained by sequence analysis of ITS region of the Trichoderma strains selected, Our results were in agreement with previous investigations on topology of Trichoderma Phylogeny (16). Furthermore, (21) employing ITS sequences for Phylogenetic analyses discriminated clades of Trichoderma section from clades of Longibrachiatum section. The results were in accordance with (22) who showed the phylogenetic trees of Trichoderma longibrachiatum recorded 98% identify with Trichoderma longibrachiatum from India, Vietnam, Australia and Malaysia.

This is evidenced through similarity matrix of Trichoderma longibrachiatum strains based on ITS analysis which was shown in Figure (15). These results match those gathered by (23) in a study where they indicated that, due to the existing polymorphisms in each taxon, the rRNA coding sequence contributed to the distinction between Trichoderma genotypes; allowing exploration of the genetic diversity, as well as establishing associations among genotypes.

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Trichoderma longibrachiatum IRAQ														
2. KY764811.1 Trichoderma longibrachiatum China	8.8													
3. KY568698.1 Trichoderma longibrachiatum Indian	9.9	7.6												
4. KJ174236.1 Trichoderma bissettii USA	7.1	7.7	5.0											
5. GQ203533.1 Trichoderma longibrachiatum Yunnan	11.0	7.2	6.9	7.8										
6. FJ571489.1 Trichoderma longibrachiatum Israel	9.0	7.9	7.3	11.8	7.1									
7. EU401554.1 Trichoderma longibrachiatum Austria	6.3	7.4	7.6	6.7	8.9	7.4								
8. KF889068.1 Trichoderma longibrachiatum Poland	10.2	7.7	8.3	8.0	4.2	7.4	6.9							
9. HE649491.1 Trichoderma longibrachiatum Saudi Arabia	7.2	11.0	7.2	7.5	8.3	10.7	7.3	8.0						
10. LC228634.1 Trichoderma longbrachiatum Japan	7.6	7.0	12.1	6.5	7.4	7.6	8.2	5.2	7.4					
11. KY346983.1 Trichoderma longibrachiatum Belarus: Minsk region	7.1	8.9	4.6	6.7	8.2	7.6	10.8	11.3	7.1	8.3				
12. LC185084.1 Trichoderma longibrachiatum Indonesia	7.0	7.2	8.0	5.7	4.2	7.0	6.1	5.8	7.0	7.0	6.8			
13. KU935695.1 Trichoderma longibrachiatum Italy	6.5	4.7	7.2	4.0	7.3	8.9	6.7	8.2	7.0	6.5	6.9	7.6		
14. KU360155.1 Trichoderma longibrachiatum Egypt	7.7	7.4	10.9	7.6	7.3	7.7	11.4	7.9	4.2	7.7	7.0	6.8	8.8	

Figure (15): Similarity matrix of Trichoderma longibrachiatum based on ITS analysis.

Conclusion

According to the results of morphological and molecular identification of the selected fungal isolate which indicate that this isolate was identified as Trichoderma longibrachiatum and recorded for the first time in Iraq as assigned AB1 strain.

Acknowledgments

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