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First New Record and Molecular Identification of *Helvella bachu* (Ascomycetes) Isolated from Iraq/Mosul

Jehaan Mowafak Al-Rawi[1] and Shimal Younis Abdul-Hadi[2]
[1]Department of Biology, College of Science, University of Mosul, Mosul, IRAQ
[2]Department of Biology, College of Education, University of Mosul, Mosul, IRAQ

[1]Corresponding Author: jehsbio88@uomosul.edu.iq

ABSTRACT

In this study, Helvella mushroom fruiting bodies samples were collected from the Nimrud area, which is located in the south of Mosul - Iraq. These samples were morphologically diagnosed based on the shape and color of the fruiting body and the shape of the ripples, while microscopically diagnosed based on the shape of cysts and ascospores, then they were molecularly diagnosed by DNA sequence data, from F- ITS1 and R- ITS4 nuclear regions. The phylogenetic analyses emphasize that there is a strongly propped lineages conformable to morphological features. As a result of our study we recorded a new rare species of Helvella mushroom in Iraq after China, then we described the distributions and ecology of the new species.

Keywords- *Helvella bachu* , Phylogeny, Helvellaceae.

I. INTRODUCTION

In general , fungi are the second largest eukaryotic organisms in nature, almost 100 000 species been described, 14000 species of which are macrofungi , most of them are edible and have a medicinal use (Meenu & Xu, 2019). Relying on research papers Ascomycota represent half number of all fungal species (Kaygusuz *et al*., 2020). The members of Ascomycota have a simple morphological shape such as Peziza sp. which characterized by a cup shaped fruit body or a large and more complex fruit body such as Helvella, Morchella and Tuber (Skrede, *et al*., 2017).

The genus Helvella, which contains many attractive species, belongs to a family Helvellaceae (Pezizales, Ascomycota) that includes nearly 52 species, it is generally known as the false morels or elfin saddles (Kirk, *et al*., 2008). This genus is widespread throughout the world, it usually grows in groups or singly in forests on calcareous soil along streams but rarely on rotting woods, it is available in abundance in spring season but rarely found in summer and autumn (Akata & Kaya, 2012). Most species of Helvella are collected from temperate regions(Wang &Chen,2002), its fruiting bodies are distinguished by different shapes like saddle, cupulate and lobed folded caps, furrowed or ribbed stipe , smooth wavy or wrinkled hymenium. The color of the fruiting bodies is also considered a distinctive characteristic, as it ranges between brown, black, gray and white, microscopically, the cysts are cylindrical in shape and contain ascospores that are oval in shape (Abbott & Currah, 1997).

In previous years, the diagnosis of fungi was based on the phenotypic and microscopic form such as the shape of the ascus and the shape of the ripples in apothecium (Landeros, *et al*. 2015). But recently when the molecular identification was established, Helvella has been extensively studied by mycologists from North America , Europe, Australia and Asia(Løken *et al*. 2020). Most studies which have included sequences of Helvella have focused on order or family phylogenetic relationships so have inserted only a few species of this genus (Zhao, *et al*. 2016).

Bachu mushroom have received great attention in recent years due to their economic importance, the yield in one year is approximate to 3000-5000 kilos , as the price of a kilogram of dried fruiting bodies in some countries reaches 150 dollars due to their delicious flavor and high nutritional value therefore, delicious soup and cookeries are made from it, which is one of the most luxurious dishes that are served in restaurants in many countries. It has many medical benefits due to the presence of a large number of biologically and pharmacologically active compounds, these benefits include antioxidant, antitumor, antibacterial, immunostimulants, and cholesterol reducing, which has attracted the interest of many researchers in this field for the purpose of developing drugs, functional foods novelty, and nutraceuticals. The truth that cannot be hidden is that “Bachu mushroom” and other mushroom types automatically used to treat many diseases such as stomachache, hyperlipidemia, and cardiovascular and heart diseases. In this context the urgency of using this amazing fungus in the development of traditional medicine must be emphasized(Badshah, *et al*.,2020). In addition to what is mentioned earlier, these fungi play an important ecological role as they form a symbiotic relationship with the roots of some trees, which is called " ecto- mycorrhizal symbionts" (Hwang *et al*. 2015). Historically, Helvella mushrooms were dried and served on the day of death holiday, it was believed that it gave life to the dead, so it is called “elixir of life” (Landeros, *et al*., 2021).

The aim of this study is a first record of *Helvella bachu* in Iraq / Mosul , it is also the first time in

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the Arab world and the second time in the world after China (Zhao, *et al*.,2016). In this paper we collected Bachu mushroom and classified it according to morphological and phylogenetic characteristics. Among the things that should be mentioned is that Bachu mushroom was previously identified as *Helvella leucopus* but recent researches has proven that there are two new species: *H. subspadicea* and *Helvella bachu* depending on the size of ascospores .

II. MATERIALS AND METHODS

1. *Specimen Collection and morphology:*

In a research trip that carries many surprises, we headed in the early morning of February, on the 16/2/2021 to the "Nimrud area" which is located 30 km south of Mosul – Iraq (Fig.1). It is one of the ancient cities of the Assyrian civilization, this area is characterized by its picturesque nature due to the passage of the Tigris River through it, the presence of beautiful lakes, and a forest swarming with willow trees. The abundance of fallen leaves that covered the ground made it a rich environment for fungal diversity. It is worth noting that the nature of the soil in this area was calcareous. The isolated fungal sample was diagnosed to the genus level using a compound light microscope at 40x magnification, the phenotypic diagnosis was made by taking part of the newly-aged pure fungal colony by "Loop" sterilized by alcoholic flame, transferred to a glass slide and mixed with a drop of dye (Lactophenol Cotton Blue) to dye the fungal hyphae and the vegetative and sexual structures.

The phenotypic characteristics of the fruiting body, ripple, texture, color and stem shape were diagnosed using the taxonomic keys found in the known taxonomic references. (Song *et al*., 2014).

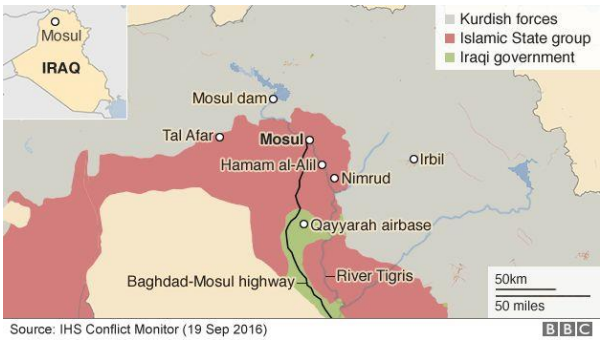

Fig. 1: (a) the map of Iraq (b) Al Nimrud area

2. *DNA Extraction Amplification and Sequencing:*

After activation of the selected fungal isolate, DNA was extracted from the fungal hyphae by an extraction kit supplied by Zymo Research Company (USA), extraction steps were carried out according to the company's protocol. To perform ITS region amplification, PCR technique was used depending on the method used by (White,1990). This process includes: Denaturation , Annealing , Extension , by using universal primers F- ITS 1 (5´ - TCCGTAGGTGAACCTGCGG-3´) and R-ITS 4 (5´ - TCCTCCGCTTATTGATATGC-3´) to determine the nucleotide sequences. A bundle of DNA was extracted from agarose gel by Gel Extraction Kit supplied by QIAGEN Company and the sample was sent to Microgen company in south Korea for sequencing . A month later, the nucleotide sequences were received from the company and compared with reference sequences deposited in the database at the global site (National center for Biotechnology Information NCBI). An alignment of the nucleotide sequences was carried out, by using the free program Blast, to obtain an accurate diagnosis of the isolate for the degree of type. Then, the degree of congruence of the isolate was investigated and compared with reference isolates that had been previously diagnosed by serial numbers in the gene bank. relying on the nucleotide sequences received from the company mentioned above and covering the ITS gene confined to the used Primer, the genetic tree was drawn using Mega 7 program(Abdulhadi, *et al*,. 2020).

III. RESULTS

1. *Identification of fungal isolate phenotypic:*

Depending on the visible phenotypic and microscopic characteristics, a preliminary diagnosis of the fungus can be made to the level of the genus. Fruiting bodies are distinguished by lobed folded caps (five irregular lobes) permanently curled edges , smooth wavy or wrinkled hymenium. The height of the hat ranges between (4.5 - 5) cm and its width is 4 cm, as for the length of the stipe is 3.5 cm and its width is 1.3 cm. The color of the hat is one of the most important diagnostic characteristics. The hat is blackish brown, the

outer surface is darker than the inner surface , whereas the color of the stipe ranges from white to cream furrowed or ribbed with hollow grooves and a noticeably enlarged base. The texture of the fruit body is smooth and soft with an attractive appearance. When a piece was taken from the inside of the fruit body and inoculated on a culture media (PDA) , the fungal colonies appeared in the form of cotton hyphae with a bright white color. The hymenium layer is described microscopically as containing a large number of cylindrical asci which contain eight oval shaped ascospores in each asci (Akata and Kaya , 2012). The dimensions of the ascospores are (16.3–20 × 10.7–13) µm. Concerning the habitat and distribution of *H.bachu* mushroom , it is generally found growing under *Populus euphratica* trees, along stream edges, in humid, shady, and sandy soil. The results were documented with a digital camera(Fig. 2).

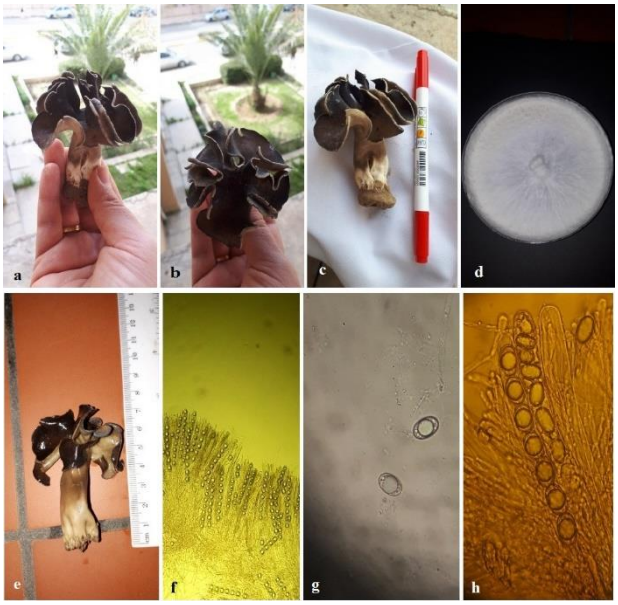

Fig. 2 : *Helvella bachu*: (a, b, c) typical mature specimens;(d) fungal colony ; (e) Length of fruit body ;(f) asci and paraphyses;(g) ascospores;(h) asci. Scale bars:(a,b,c,e)1 cm , (f,h) 40 µm, (g) 20 µm.

2. Molecular identification of the fungal isolate:

It was possible to simulate the replication of genetic material outside living systems, with some differences, using the PCR technique, which is the modern reference method and the technology adopted in scientific research laboratories, during which many copies of small pieces of DNA molecule are made on a logarithmic or incremental scale. After extracting the DNA and containing the region to be amplified based on specialized primers (ITS1 and ITS4), which are carefully selected so that the primers are not duplicated and act as a starting point for elongation, the reaction is detected by electrophoresis on agarose gel for most of the polymerase chain reactions. This reaction provides the possibilities to identify the movement of the DNA molecule, such as the position of the bundles in each path, and by using ethidium bromide dye that has the ability to shine when binding with the DNA molecule, which is only shown using ultraviolet rays to show a single horizontal band of clear brightness with a size of 650 base pairs. Then, the bright bundle was compared to a standard scale or molecular marker that contains several pieces of a known size. After that, a digital camera was used to photograph the bright bundle, and the photographing process was repeated several times to reach the best picture, as shown in the figure(3).

The result obtained confirms the success of the polymerase chain reaction which depends mainly on the target to be amplified, the components of the reaction buffer, the concentration of the mixture components, the accuracy of the designed primers, the presence of a correlation between the primers content of G.C, the number of cycles used, and the absence of contaminants from DNA molecules and the devices and tools used.

The literature in this context indicated that two new isolates of ascomycetes Helvella were recorded in western China, *Helvella bachu* and *Helvella subspadice*, based on the polymerase chain reaction technique for gene amplification ITS4 and ITS5 (Zhao, *et al*. 2016). According to an extensive study conducted in Europe by Skrede *et al.* (2017), seven new species were identified among 55 species belonging to the genus Helvella: H. alpicola, H. alpina, H. carnosa, H. danica, H. nannfeldtii, H. pubescens, and H. scyphoides depending on the ribosomal DNA rDNA and using PCR technique. In a study conducted by a research team led by Kaygusuz *et al.* (2020) which was based on the amplification of the ITS rDNA gene region by using PCR technology, the relationships and genetic variations of two strains of the genus Helvella: *Helvella fibrosa* and *H. macropus*, collected from Turkey, were identified.

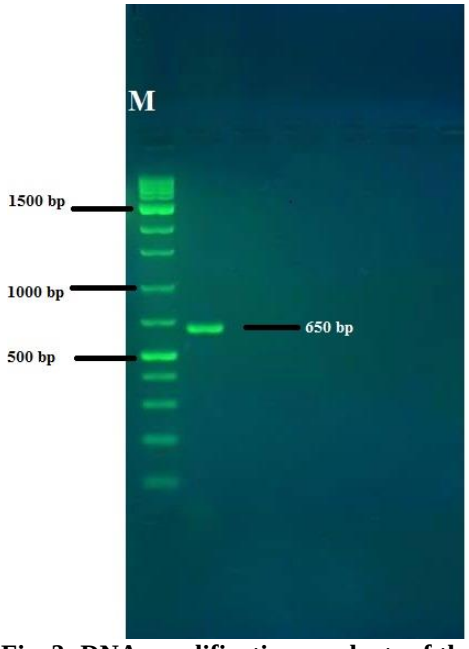

Fig. 3: DNA amplification products of the fungal isolate on agarose gel

3. The nucleotide sequence of the fungal isolate:

After completing the polymerase chain reaction and amplifying the desired target by using a special extraction kit, the DNA bundle was cut from the agarose gel and placed in an eppendorf tube , then the forward primer and non-ionic water were added to the tube, after that it was sent to Macrogene Company in Korea to find out its nucleotide sequence. After three weeks, the results came in the form of a fasta file as shown in figure(4).

CCGGGACTGGCGAGGACCCTAGACCCACCGGAC
AGTGGTT
GGAGCGCCCGAAGGTTCGGCTGACACCGGACGC
GGCGCGCTCGCCCAGCTCCCGCCCTTCAACCGC
CGGGAGGCCAGTTTCCAACGCCAACTCTCTGCG
TACCTCTCCACTGTTGCTTCC
CCGGGGGGTCCCTCATCCCCCGGGGGAGGTCCC
CGAGCAA
GACGCGCCGCCAAACCCAACGGCGGCCCCATCC
GTCTGAC
GGCCAGCGCGCCGCCGAGGAAGCGGAAGCGAC
CGACGAAGCTGAAATACGAAAACAAAGAAAAC
TTTCAACAACGGATCTCTTGGTTCCTGCATCGAT
GAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCT
TTGAACGCACATTGCGCCCCCTGGCATTCCGGG

Fig. 4: The nucleotide sequences of the local fungus isolate *Helvella bach*

The nucleotide sequences were deposited directly into the gene bank, which contains a comprehensive, publicly available, free annotated database of references and active information. Alignment of the nucleotide sequences of the selected isolate was carried out using Blast search, which was a set of programs available on the NCBI website (http://www.ncbi.nlm.nih.gov/blast) that provides the search for regions of identity or similarity between the entered sequences and the sequences deposited in the gene bank and comparing them with each other in order to know the identity of the organism. The alignment process showed that the nucleotide sequence of the local fungal isolate belongs to the species *Helvella bachu*, which showed a 99% agreement with the data deposited for the nucleotide sequences of the reference strain from China *Helvella bachu* HKAS 88105 affiliated under the sequence ID reference number: NR_155171.1. By clicking on the serial number, the data for heterogeneity at the level of five nitrogen bases appeared, as shown in Table (1) and figure (5).

Table (1) the serial number of the reference strain from China, the percentage of its congruence with the selected local isolate *Helvella bachu* and the data of the variance with this isolate.

small subunit ribosomal RNA gene, partial							
No.	**Type of substitution**	**Location**	**Nucleotide**	**Sequence ID with compare**	**Sequence ID with submission**	**Source**	**Identities**
1	**Transition**	**121**	**G\A**	**NR_155171.1**	**OL454919.1**	***Helvella bachu***	**99%**
	Transvertion	**133**	**A\C**				
	Transition	**203**	**C\T**				
	Transvertion	**371**	**C\A**				
	Transition	**435**	**C\T**				

Helvella bachu HKAS 88105 ITS region; from TYPE material

Sequence ID: NR_155171.1Length: 779Number of Matches: 2

Range 1: 106 to 538GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
759 bits(841)	0.0	428/433(99%)	0/433(0%)	Plus/Plus

Query 1 CCGGGACTGGCGAGGACCCTAGACCCACCGGACAGTGGTTGGAGCGCCCGAAGGTTCGGC 60
Sbjct 106 .....**G**.....**A**..... 165

Query 61 TGACACCGGACGCGGCGCGCTCGCCCAGCTCCCGCCCTTCAACCGCCGGGAGGCCAGTTT 120
Sbjct 166 .....**C**..... 225

Query 121 CCAACGCCAACTCTCTGCGTACCTCTCCACTGTTGCTTCCCCGGGGGGTCCCTCATCCCC 180
Sbjct 226 285

Query 181 CGGGGGAGGTCCCCGAGCAAGACGCGCCGCCAAACCCAACGGCGGCCCCATCCGTCTGAC 240
Sbjct 286 345

Query 241 GGCCAGCGCGCCGCCGAGGAAGCGGAAGCGACCGACGAAGCTGAAATACGAAAACAAAGA 300
Sbjct 346 .....**C**..... 405

Query 301 AAACTTTCAACAACGGATCTCTTGGTTCCTGCATCGATGAAGAACGCAGCGAAATGCGAT 360
Sbjct 406 .....**C**..... 465

Query 361 AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 420
Sbjct 466 525

Query 421 CTGGCATTCCGGG 433
Sbjct 526 538

Fig. (5) The nucleotide sequences of the reference isolate confirmed in Gene bank with the sequence number NR_155171.1 showing the sites of heterogeneity with the local isolate *Helvella bachu*

According to the alignment process of the nucleotide sequences of the fungal isolate, after linking it with the databases in NCBI and using the registration number, it was recorded for the first time in Iraq, specifically in the city of Mosul, in the database of the gene bank with the reference serial number OL454919.1 . The registration process has been approved depending on the accurate entry of the nucleotide sequence and coding regions, the region where the fungal isolate was taken from, the researcher's affiliation point (Mosul University) and the date of sample collection. The officials in charge of the gene bank gave the sequence submission the accession number two days after receiving and reviewing it, confirming the success of its deposit in the gene bank database and the correctness of its classification. The draft registration was returned to the researcher for review before entering the database.

There was plenty of time to search the database to make it clear that the isolate is recorded for the first time in Iraq and the Arab world, and the second isolate is recorded worldwide after the Chinese isolate.

4. Phylogenetic tree:

The genetic tree (Dendogram) was drawn by using the Mega program version 7, which is freely available on the NCBI website, then it was divided based on the results of the nucleotide sequencing of the local fungus *H.bachu* isolate into two main clusters, as shown in figure(6). The first main cluster was divided into two secondary clusters, the first secondary cluster included the Chinese species deposited in the gene bank with serial numbers KU739794.1, KU739793.1, and KU739795.1, which were 100% identical. The second secondary cluster included the Chinese species deposited in the gene bank with the serial numbers NR_155171.1 and KU739791.1, with a 100% congruence rate. As for the second main cluster, it included the Iraqi local isolate, which was recorded in this study with the serial number OL454919.1 with a matching percentage of 99%.

Previous literature indicated that PCR technique and analysis of DNA amplification products play a major role in diagnosing fungal species with high accuracy, especially species that are closely related in phenotypic traits, and contribute significantly to the detection of fungal species of medical and industrial importance.

3 species of Helvella mushroom were classified by Zhao *et al.* (2016) which collected from(Jiuzhaigou) natural reserve southwest China using ITS region and 28S rRNA gene sequencing, two of them recorded for the first time in China: H. ulvinenii and H. subglabra.

In the same context, Kaygusuz *et al.* (2020) indicated that the ITS region-specific primers have a significant role in the identification of species belonging to the genus Helvella to determine the genetic and evolutionary relationships of Turkish species of the genus Helvella.

Landeros *et al.* (2021) also relied on ITS and LSU region to classify a new species belonging to the genus Helvella, which was *Helvella jocatoi*, for the first time in the middle of the forests of Mexico (Abies religiosa).

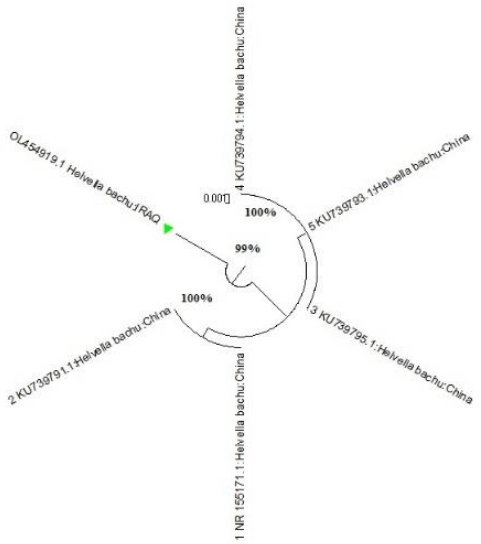

Fig. (6) Phylogenetic tree of the local isolate *Helvella bachu* and global isolates

Table (2) Genetic dimension of the local isolate *Helvella bachu* isolate and global isolates

	1	2	3	4	5
1. OL454919.1 Helvella bachu:IRAQ					
2. 1 NR_155171.1:Helvella bachu:China	0.0117				
3. 2 KU739791.1:Helvella bachu:China	0.0117	0.0000			
4. 3 KU739795.1:Helvella bachu:China	0.0141	0.0023	0.0023		
5. 4 KU739794.1:Helvella bachu:China	0.0141	0.0023	0.0023	0.0000	
6. 5 KU739793.1:Helvella bachu:China	0.0141	0.0023	0.0023	0.0000	0.0000

Table (3) Genetic similarity ratios for the local isolate *H.bachu* compared to the reference sequences global loaded in the NCBI

	Accession	Country	Source	Compatibility
1.	**ID: NR_155171.1**	**China**	Helvella bachu	**99%**
2.	**ID: KU739791.1**	**China**	Helvella bachu	**99%**
3.	**ID: KU739795.1**	**China**	Helvella bachu	**99%**
4.	**ID: KU739794.1**	**China**	Helvella bachu	**99%**
5.	**ID: KU739793.1**	**China**	Helvella bachu	**99%**

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