First recording and molecular diagnostics of three ascomycetous macrofungi from Nineveh, Iraq

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Abstract

This research project has referred to three of macrofungi (Ascomycota): *Geopora foliacea*, *Helvella leucopus and Peziza vesiculosa*. The first two types of macrofungi have been collected from Al- Nimrud area ،While the third type (*Peziza vesiculosa)* was collected from Mosul Dam northern of Mosul. The samples were diagnosed phenotypically and microscopically, as their morphological and microscopic characteristics were determined. Moreover, the samples were molecularly identified by DNA sequencing data. These macrofungal species are recorded for the first time from Iraq.

Keywords: macrofungi, *peziza vesiculosa*, *helvella leucopus*, dna sequence

Introduction

In recent decades, there has been an increased interest by researchers in macrofungi, which are considered the second largest eukaryotic organisms in nature. These macrofungi are a rich food source in nutrients, in addition to their benefits in the medical and pharmaceutical fields (De Silva, *et al*., 2013) [6]. Ascomycetous Macrofungi are all fall into one phylum, which is called phylum Ascomycota. They are the largest fungal phylum because it includes 15 classes, 68 orders, 327 families, 6500 genus, 64-93 thousand diagnosed species, and many species which have not been diagnosed yet. Thus, they constitute 43-63 % of the total number of the diagnosed fungal species. In addition, they are considered the most diverse and widespread eukaryotic organisms on earth (Wijayawardene, *et al*., 2017; Senanayake, *et al*., 2022) [18, 15].

Pezizomycotina is considered the biggest subdivision in Ascomycota, which include many orders some of them is Pezizales (Kirk *et al*., 2008; Schoch *et al*., 2009) [11, 14]. The species of this order are characterized by the formation of asci with an outer cover, operculum, which opens at maturity to release the ascospores from it. Whereas their fruiting bodies maybe in a cup-shaped, like the species of the type *Peziza*, or the fruiting body maybe larger, and more complex as in the species of *Morchella* and *Helvella* (Hansen *et al*., 2002; Skrede *et al*., 2017) [8, 17]. Some members of this order are considered economically important, such as truffles, morels, and the genus *Morchella* as well (Hansen, *et al*., 2005) [9]. Among the families of this order is the family pezizaceae, which includes 230 species distributed into 31 genera, and the genus Peziza is the largest which includes 80-104 species (Kirk, *et al*., 2001; Spooner, 2001) [10].

Al- Nimrud is a district in Nineveh Governorate, north of Iraq (Fig. 1), which is 37 km southeast of Mosul city (220 m above the sea level) and 327 km north of Baghdad, the capital of Iraq. Al- Nimrud district is rich deciduous leaves from *Populus euphratica*, in addition to its closeness to the Tigris River, which makes it a rich environment for fungal diversity, and the growth of a wide range of large cystic fungi.Mosul Dam (Sad Al- Mosul) is located on the Tigris River (Fig.1), 60 km northwest of Mosul city, 228 m above sea level, and 395 km north of Baghdad (Al- Ansari, *et al*., 2021) [2]. It is one of the areas with heavy rain in winter, and with high humidity. These areas are rich in large number of fungi yet, still unexplored.

During the field research and for different periods, two samples of cystic fungi were collected from Al- Nimrud district, and one sample from Mosul Dam.

In this research, a description of the three cystic fungi: *Geopora foliacea*, *Helvella leucopus*, *Peziza vesiculosa*, and based on their morphological characteristics and molecular diagnosis, in order to record these fungi as one of the large cystic fungi recorded for the first time in Iraq.

Fig 1: (a) Map of Iraq showing Nineveh Governorate, (b)Al Nimrud District, (c) Mosul Dam area

Materials and methods

1. Specimen collection and morphology

Macrofungi samples were collected from various locations in Al- Nimrud district and Mosul Dam within Nineveh Governorate during the periods (February- May). The fruiting bodies were found among the fallen leaves singly, and some were found in the form of convergent. The phenotypic characteristics were determined in which a study was conducted on the size of the fruiting body, the shape, color, texture, the cap color and dimensions, the outer and inner surface, and its color.

In addition to that, the microscopic characteristics (observing the asci and the number of the ascospores inside, the dimensions of the asci, its shape, its starchy top whether it takes iodine dye or not, the shape of the sack end, ascospores, its dimensions and shape, the presence of oily drops or not, the dimensions of the paraphyses, and their shape), in addition to the season of the appearance of the fruiting body samples. The samples were photographed in their natural environment and in the laboratory. The lactophenol dye was used in order to dye the different parts of the obtained isolated fungal to be examined using a compound light microscope, and kept in Formalin Acetic Acid (FAA). The examined samples were stored in the Department of Science, College of Education for Pure Sciences, University of Mosul, Iraq.

2. DNA extraction amplification and sequencing

After collecting the fungal colonies in a pure form, the DNA was extracted from the fungal hyphae using an extraction kit provided by Zymoresearch (Catalog No. D6005), and using the ITS gene. Also, Polymerase Chain Reaction (PCR) was conducted under the following conditions: Denaturation 1 at 95 °C for 5 minutes, Denaturation 2 at 94 °C for 30 minutes, Anealing at 56 °C for 30 minutes, Extension at 70- 72 °C for 30 minutes, Final extension at 70- 72 °C for 5 minutes.

In order to determine the nitrogenous bases sequences for each fungus, the result was sent the Korean company (Macrogene). The result was received for nucleotide sequences, and was compared with the reference sequences in the database of the National Center for Biotechnology Information (NCBI).

Through the Blast program, an alignment of the nucleotide sequences for accurate diagnosis was made to the rank of the species. And to indicate the degree conformity with the other reference isolates of the same genus that was previously diagnosed and registered in Gene Bank. Based on the nucleotide sequences within the used initiator, the Phylogenetic tree was separately drawn for each fungal isolate after using Mega 6 program.

Results

1. Phenotypic and Microscopic Diagnosis: According to the taxonomic keys mentioned in the scientific references, a phenotypic and microscopic diagnosis of collected fungi was conducted as *Geopora foliacea.*

2. Macroscopic features

Fruiting body (apothecia): cap: 3-5 cm wide, spherical, completely immersed in the soil, when ripe, it appears above the soil with a hole that has cracked edges. The inner surface (fertile layer): light creamy, smooth, and shiny. The outer surface (sterile): brown, and smooth.

3. Microscopic features

Asci, 225x15-22.5 µm, cylindrical, Inamyloid (ascus cover does not take iodine dye), 8 – spored. Spores: 25-30 x 12.5-17.5 µm, ellipsoid, transparent, contain a large circular, oily drop. Paraphyses: 250x12.5-20 µm, cylindrical. edibility unknown, The Fruiting time is February- April. (Figure. 2) shows the phenotypical and microscopic characteristics for the fungus.

The description mentioned above corresponds with: Ahmed (1978) [1], Peric (2002) [12], Peric & Peric (2011) [13]. *G foliacea* is considered the second registered fungus after the G.arnecolla type (Al-Khesraji & Suliaman 2019) [4].

Fig 2: *G. foliacea*. a, b: fruiting body in natural habitat, c, d: fruiting body in lab, e. hymenium, f.: asci with spores g & h: ascospores i. paraphyses with ascus. j, k, l: skeletal hypha

Helvella leucopus

Macroscopic features

Fruiting body: cap: 2.5-4 cm, saddle-shaped that includes 3-4 lobes, wavy.

The inner surface (fertile): light hazel color, smooth, the edge is flat.

The outer surface: dark brown, smooth, matte; stipe: 4.5 - 7 x 1 - 3, white, hollow, widened at the base.

Microscopic features

asci 250 – 300 x 17.5 -20 µm with eight cystic spores, non-starchy, with vesicle end, 8 – spored.

spores: 17.5 x 12.5 µm, oval, transparent; paraphyses: 280 – 300 x 12.5 µm, cylindrical.

It is found singly, the fruiting body is found on the soil rich in deciduous leaves, inedible. The fruiting time is February- May. (Figure.3) shows the phenotypical and microscopic characteristics for the fungus.

This type of fungus is added to the seven types of the same genera that are registered in Iraq (Al- Khesraji, 2016) [5]. In a study conducted by (Ge *et al*., 2022) [7] on mice diagnosed with hyperlipidemia, it was evident that polysaccharides (HLP) which was prepared from *Helvella leucopus* by hot aqueous, alcoholic extract of the fungus had anti- hyperlipidemia effect and decreased total cholesterol, triglycerides, and low lipoprotein cholesterol, and density in the blood serum. These sugars have the ability to improve the metabolism of fats in the blood.

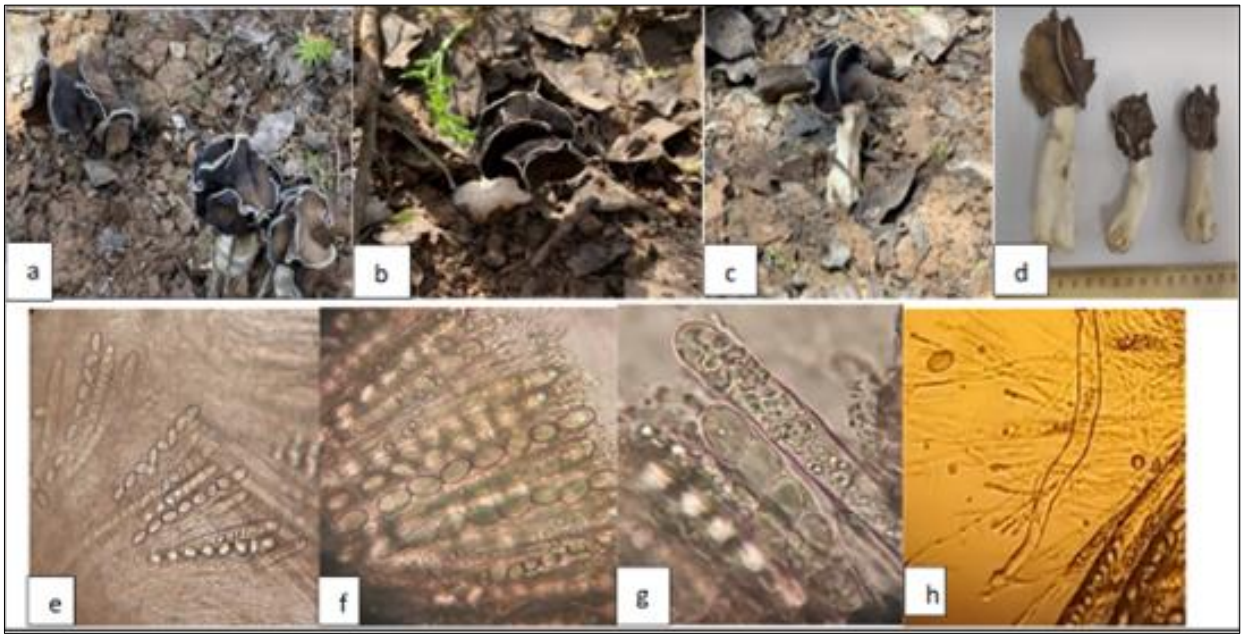

Fig 3: *H. leucopus*, a, b, c: fruiting body in natural habitat, d: fruiting body in lab. e-g: paraphyses with ascus. h: the lower end of the ascus is vesicle.

Peziza vesiculosa

Macroscopic features

Fruiting body (apothecia): cap: 2-3 cm, the fertile layer (upper surface), brown, smooth, the lower (outer) is light hazel, smooth, the edge is flat.

Microscopic features

Asci 270-275 x 15-17.5 µm, 8-spored, obtuse lower end, amyloid (the ascus cover take iodine dye). spores: 10-12.5 x 5-7.5 µm, elliptical, smooth, without oily drops.

Paraphysis 280-300 x 12.5 µm.

They are found in clusters, and few are single. The fruiting body is found on the soil under the fallen leave; inedible. The fruiting time is April – May. This type is considered the second from *Peziza* genus that is registered in Iraq after *P. proteana* type (Al- Khesraji, 2018) [3]. (Fig. 4) The morphological and microscopic characteristics of the fungus.

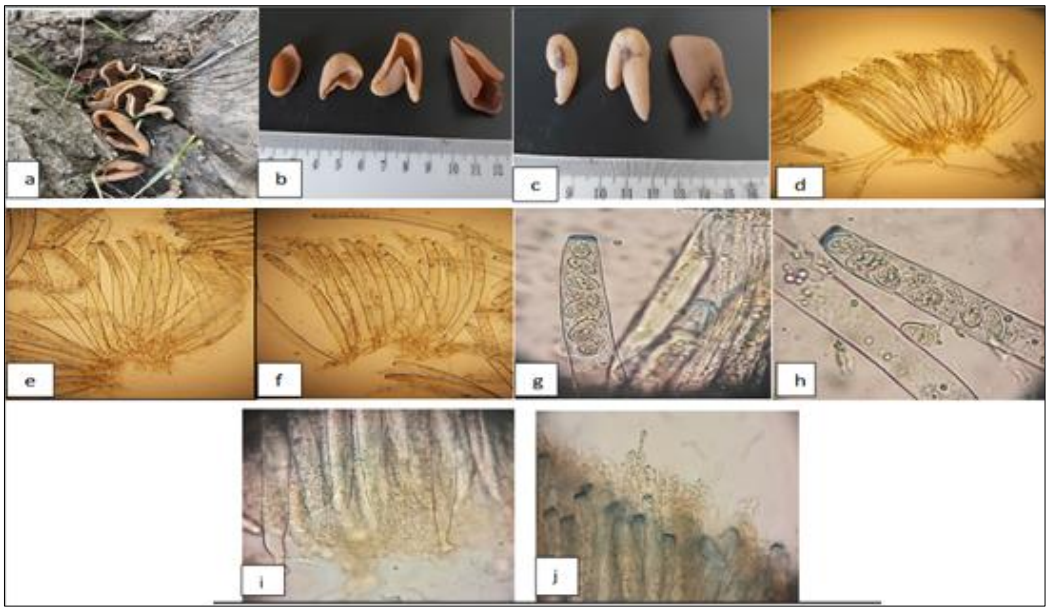

Fig 4: *P. vesiculosa.* a: fruiting body in natural habitat, b, c**:** fruiting body in lab. d-f: hymenium, g, h: asci with spores i: lower end of ascus is obtuse. j: Paraphysis with ascus.

Molecular diagnosis of the obtained ocal fungi

The outcome of the extracellular polymerase chain reaction (PCR) after the completing the electrophoresis process revealed sparkling bands when spotted under ultraviolet light, in which the bromide strongly connected to the DNA, especially in the large groove, gives radiance to the horizontal bands with distinctive brightness. Its molecular weights ranging from 650-640 base pairs compared to the standard scale ladder that consists of a series of pieces of different sizes used for comparison (which was documented with a digital camera where many pictures were taken to select the best shot, as shown in Figure (5).

The results collected shows the success of the polymerase chain reaction (PCR) which mainly depends on the target model to be amplified, the reaction buffer, the concentration of the mixture components, the accuracy of the primers design, in addition to the existence of a correlation between the content of the initiator of G.C, the number of cycles used, and the absence of DNA molecules, and the devices and tools used from the contaminants.

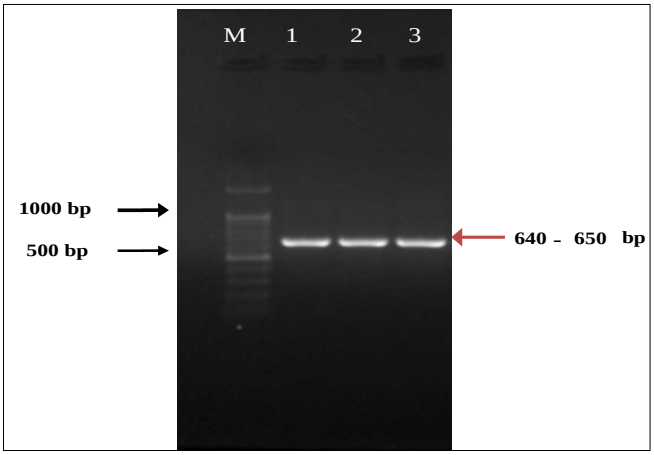

Fig 5: DNA amplification results of fungi obtained on the agarose gel where M path symbolizes: a size marker, lanes 1 -10 are nucleic acid amplification results, and nucleotide sequence of isolated fungi

The nucleotide sequence of the fungal *G. foliacea*

After the process of amplifying the target gene ITS, the result of the amplification was sent to the Korean company Microgen to confirm the fungus identity through finding the nucleotide sequences. The results came back from the company after three weeks in files of several formats that confirms what was previously stated in the phenotypic diagnosis as shown in Figure (6).

>H220504-023_O21_A4_AF.ab1 1060
CATTAGTGGATGACATGTTTCGCAGCATGACATTTCAAATCCCACCCGAGTATCTTA
CCCGTTGCTTCCGTGCCGCACACGCCACCAAGGTGTGCCTCCTGGTCTTGCGGTACC
TAGTATCGCTGGCCCGTGGGGAGCCGGCACGGGAGGTTAACCCCAAACTCTTGCCTT
CCATTGCCTTCCGTCTGAACTGTTAGTACATGAAAAGTTAAAACTTTCAACAACGGA
TCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATT
GCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCTCCTGGTAATCGG
GGAGGCATGCCTGTCCGAGCGTCACTAAAATCAACTCAAGCATTTCTGTGCGTGGTC
ATGGAGGAAGAGTCGGTCTCGTTGCAGCCCGTCTCCCCTCCCAAAATCAAAGGCGG
AAGGTCACTGGTGCTCTGCGTAGTAGTATTGTTTCGCTGACATTCCAGTGTCTCTTCG
CGCCTCCAAACCCCCACAATTCTCTTGATTGACCTCGGATCAGGTAGGGATACCCGC
TGAACTTAAGCATATAAACCCGGGGGAAAAAAAAATCCCCAGTGGAAGAAATAGCC
CGAACAGGAAAAGCCAAACCCCCCCCGGGGCTACCCCCAGAGAGGGCCCCCACCCC
CCACCAAGGAGGCCCCACTGCGCAGGGAAACAAAAAAACCCGGCCCCCGGGGGGG
GAACGGAAAAATAAACCAAGAACCCTGCCTCCAAAGACCTCCCGCACAAAAATAAA
AAAAACAAAGAAGAAAAAAAACCACCATAAACACCGATCCTCCCCAAAAAAAAAA
ACGACACACGCAAGATGGGGGGAGAGAGGAATAGCACGCAACACACACAACCCTA
CCAGACAACACCCCCCCCGCCCGACAGGAGAGAGAAGACCCCCTCTCGCGCCCCCA
GAAAAAAAAAGAAACAAAGAAGAGTAGGGTGGGGCCGTAGAAAGAAGAAAAACA
CATACCTCTCCCCGCGCCCCCCCTCCCCCCCCATACAAAAAGTGGGG

Fig 6: Nucleotide sequences of local fungus, *G. floacea*

The research paper provides identity and similarity areas between the introduced and the sequences deposited in the Gene Bank, then compare them for the aim of finding the identity of the organism.

The results of the nucleotide sequencing process of the local isolated fungus (after being listed on the NCBI website http://www.ncbi.nlm.nih.gov/blast) showed that the nucleotide sequence of the local isolated fungus belongs to the type *G. foliacea*, which was later registered in US Gen Bank with the identification number ON667912.1 Accession number, and for the first time in Iraq after required information input as the collection date, the place of collection, the researchers affiliation, and reviewing the information by Gen Bank team.

Multiple sequence alignment

The nucleotide sequence analysis technique was conducted for the local fungus *G. foliacea* and was compared with the rest of the strains taken from parts of the world. They were previously deposited as reference isolates on the NCBI site. The results showed the presence of some point mutation in some isolates or as called single nucleotide polymorphism (SNP) distributed among various isolates.

The local isolate showed a 99% match with the data deposited "submission" for the nucleotide sequence of the Spanish reference under Sequence ID: JN812046.1 and it was evident after clicking on the serial number, which showed the data of the variation that resulted from a presence of a point mutation at the level of one nitrogenous base at site 292, which is a type of Transition that led to conversion of cytosine into thymine as shown in the table (1) below:

Table 1: Genetic similarity ratios for the local fungus *G. foliacea* compared to the reference sequences global registered in the NCBI

	Accession	Country	Source	Isolation source	Compatibility
1.	ID: JN812046.1	Spain	*Geopora foliacea*	----------	99%
2.	ID: FM206432.1	Estonia	*Geopora sepulta*	----------	91%
3.	ID: KU991184.1	USA	*Geopora sepulta*	----------	91%
4.	ID: KU924372.1	France	*Geopora* sp.	ectomycorrhiza	90%
5.	ID: DQ200831.1	Norway	*Geopora cf. cervine*	----------	89%

It is worthy to mention that the registered or known species do not exceed 1% of the number of species estimated to exist on planet earth.

Phylogenetic tree

Based on the nucleotide sequence data of the fungal isolate, the sequence obtained from Schukos process was entered into Mega program, version 6, which is freely available on the NCBI website. The evolutionary tree was drawn using the UPGMA method, which was built with a total length of branches estimated by 382.30652239, within the fixed scales with branch lengths in the same units and evolutionary distances used to the phylogenetic tree with source samples. Evolutionary distances were calculated by using (Maximum Composite Likelihood Method). Which is based of switching bases at each site on the gene to be examined. The analysis

included (5) isolates in addition to the local isolate to be examined and to know the percentage of genetic conformity.

Based on the results of the analysis of the evolutionary tree that branched into two main clusters, the first branch was divided into two secondary branches. It was found that first branch of the cluster is the nearest homolog species of the local isolate. The isolate carries the serial number (JN812064) with a matching ratio of 99%. While the second group of the first cluster included the reference isolate with serial numbers FM206432.1, KU991184.1, and it showed genetic match by 91%.

In addition, the second cluster included reference isolate with serial numbers KU924372.1 as shown in Table (1). DQ200831.1,

Many fungi evolutionary relationship have not yet discovered due to several factors, some of which:

1. Complex diversity of fungi in their way of living.
2. The presence of groups of organisms that have been treated as fungi and for a long time. This is due to the similarity of their reproductive structures, and environmental behavior.

Therefore, the completion of drawing the fungi evolutionary tree still need in-depth research efforts to parallel the expanded taxonomic data.

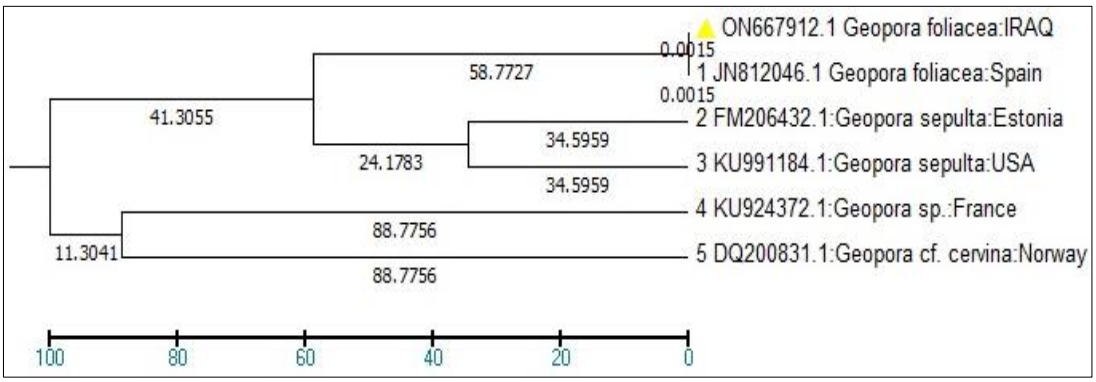

Fig 7: Genetic affinity tree of the local fungus *G. foliacea*, and the reference global isolate

>H220526-021_I05_A14_AF.ab1 850

CATCGGACAGGACAGATTCAGGAACACGGGAGGGCGTGGGGTAGCCCGACCGGGTTTGAGCCTCCCG
GGACTGGCGAGGGCCCTAGACCCAACGGACAGTGGTTGGAGCGCCCGAAGGTTCGGCTGACACCGGA
CGCGGCGCGCTCGCCCAGCTCCCGCCCCTCAACCGCCGGGAGGCCAGTTTCCAACGCCAACTCTCTGCG
TACCTCTCCACTGTTGCTTCCCCGGGGGGTCCCTCATCCCCCGGGGGAGGTCCCCGAGCAAGACGCGCC
GCCAAACCCAACGGCGGCCCCATCCGTCTGACGGCCAGCGCGCCGCCGAGGAAGCGGCAGCGACCGAC
GAAGCTGAAATACGAAAACAAAGAAAACTTTCAACAACGGATCTCTTGGTTCCCGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCCCCTGGCATTCCGGGGGGCATGCCTGTTCGAGCGTCTCTGGGACGAACACCCTCGCGCAAGGTGG
ATGCGCGGTCTTGGCGGCGGTGGCGTGCACGACGAGGTGGCCACCGGGCTGAAATCAATGGGCGGAC
GCCTGCCGCGTGGCCGAGCGTGATAAGGCGAAAGTGCGCCAGCGCGCGGCGAGGCTGCCAGCCATAG
CCCGTCCTCTCCCGGACGGGGGGAGAAAAAAAACATCGGAACTCCTCGAATCAGGCAGGGATACTCGC
TGACTTAAGCATCATAATAAAACAGCCGAGAAAGAGGAGAAGGGATCATTTACAGAACCAAAGAACCA
AGATTCCCGGAAAACACCGGGAAGGGGCTTGGG

Fig 8: Nucleotide sequences of the local fungus *H. leucopus*

The nucleotide sequence process of the second local isolate showed that it belongs to the type *H. Leucopus*, which was registered in the US Gen Bank with the ID serial number: ON667912.1 Accession number, and for the first time in Iraq, and the Arab world. And after entering the all the required information as previously stated, and after reviewed by the Gen Bank team. Also, after conducting a multiple match analysis at a rate of 1.1 to 588, which represents the sites taken from the source gene "subject", which was compared with the local isolate gene "Query", the local isolate showed a high match (identities) of 99%, 568/ 588 and a score rate of approximately 1052/1166 with the nucleotide sequences for the Chinese isolate reference (Shanghai) deposited in the Gen Bank with ID: KC137337.1.

Also, it was shown that after clicking on the identification serial number, it was evident that there was a variation resulting from two- point mutation, at the level of one nitrogenous base at site 132, which is from type Transition, that led to conversion of Thymine into Cytosine (T to C), shown in table (2).

It is important to state that changing one base may lead to the production of heterogeneous amino acids that are not required for the living cell. And this leads to the production of defective protein or production of the same amino acids, and it will be silent mutations or may lead to stopping the production of an amino acid as a result of translating it into (Stop Codon) as it is called non- sensible mutations.

Table 2: Genetic similarity ratios for the local fungus *H. leucopus* compared to the reference sequences global registered in the NCBI

	Accession	Country	Source	isolation_source	Compatibility
1.	ID: KC137337.1	China:Shanghai	*Helvella leucopus*	Tree	99%
2.	ID: JX462565.1	Italy	*Helvella leucopus*	----------	92%
3.	ID: JX462574.1	China:Yunnan	*Helvella leucopus*	----------	90%

Phylogenetic tree

The results of drawing the genetic affinity tree, which was built with a total of branches length estimated by 8.83170789 and which was drawn with fixed scales with branches lengths in the same units, revealed the evolutionary distances used to build the phylogenetic with local isolate. Also, the evolutionary distances were calculated using the Maximum Composite Likelihood Method, which based on the process of changing the bases at site of the gene to be examined. The analysis included (3) isolates in addition to the local isolate and to know the percentage of its genetic affinity.

Also, based on the analysis results of the genetic evolutionary tree, which was divided into two main clusters. The first cluster which was branched into two secondary clusters, included the most closely related types to the local isolate, which is the Chinese isolate with the serial number KC137337.1

In which the two isolates showed a similarity of 99%, whereas the second cluster showed that the local isolate showed less affinity when compared with compared with each of the isolates with the serial numbers JX462574.1 and JX462565.1, in which these isolates showed a similarity of 92% and 90% respectively, as shown in table (2).

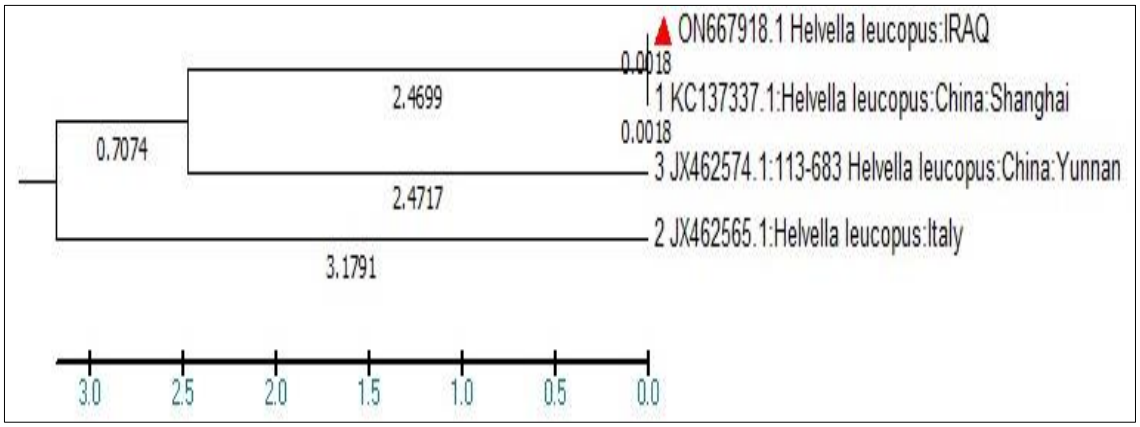

Fig 9: Genetic affinity tree for the local fungus *H. Leucopus* and the reference global isolate.

>H210819-062_G17_V11_VF.ab1 682

CAGGAGCCGATCACACACCCCATTGTCTACCATTACCATGTTGCTTCCACTGGACAGGCCGGACCCCTCT
TCTTCAATACTGAAGGCGGGAGGTTCAGCCCTCTGGCCATTCGACCTTAAAAATCGAACAGCTGGGGAG
TGCCGGTGGATGGCCCCCTTAAAAAAACTTTTTAAAGAAGCATTATACGTCTGAACTACTGTTTTATAAC
AAGAAAACATTATAAAACTTTCAACAACGGATCTCTAGGCTCTTGCATCGATGAAGAACGCAGTGAAAT
GCGATACGTAATGTGAATTGCAGAATCTCGTGAATCATTGAATCTTTGAACGCACATTGCGCCTTATGGT
ATTCCATAAGGCATGCCTGTCTGAGCGTCAGGTCCCCCCCACTCAAGCACCTTTTTTTAATCCAAAATCGT
GCTTGGATTATTTTGGATGAGCAGCTGTCAAAGGCTGCTGTCCATAAATTCATTGGCAGTATGGTTTGTT
CTTTCCAGGCTGAGCGTGAGAAATTTACCCTTACCCCGCTCCATTATGGATTGGAATTGAAAGTCGCCCT
TACCCCCCCAATATCAAGCAACTTAAGTAAAGTTTTTGTTTATTTTGGGGTGACCACCGGATTCAGGGCG
AGCTTATCCCGTTAAAACTCAATCTTGAACCCCCCCGGGGAAAGAAAAAAAA

Fig 10: Nucleotide sequences of the local fungus *P. Vesiculosa*

As shown from the result of the amplification nucleic acid, it appeared that there is a sparkling buddle with molecular weight of 650 base pairs, the result of the nucleotide sequences came after performing the line-up process at the NCBI site, to confirm what was revealed by the phenotypic diagnosis and its connection to the type *Peziza Versiculosa*. The isolate was registered in the Gen Bank with ID: ON667914.1 Accession Number and for the first time in Iraq after reviewing the nucleotide sequence and all information related to the fungal sample by the Gen Bank team.

The technique of multiple match analysis for the fungus isolates when comparing the nucleotide sequence with the rest of the strains that are deposited in the Gen Bank as reference isolates revealed that there are multiple point mutations. This

was in the results of local isolate with the Egyptian isolate with ID number: MT792532.1.

Also, there are (4) types of point mutations distributed in different sites.

The first one in the site 374, type Transversion, which led to the conversion of cytosine into guanine (C to G), while the second is located in site 393, which is from type Transition that changed thymine to cytosine (T to C).

The third one is located in site 399 which also from type Transition that led to changing cytosine into thymine (C to T), whereas, the fourth is from type Transition as well. It is located in site 503 and it changed thymine into Guanine (T to G). The genetic matching rate is 99% as shown in table (3).

The comparison was also conducted with the Italian isolate with the ID: JF908568.1, which there were (4) point mutations distributed in several sites in which the first one is located in site 374 and it is from type Transversion that led to converting cytosine into guanine (C to G), while the second is available in site 393 and it is from type Transition that changed thymine to cytosine (T to C).

The third is in site 399 and it is from type Transition that changed cytosine into thymine (C to T). While the fourth one is in site 503 and it is from type Transversion that converted thymine into guanine (T to G). The genetic match rate was 99%.

Tab 3: Genetic similarity ratios for the local fungus *P. vesiculosa* compared to the reference sequences global loaded in the NCBI

	Accession	Country	Source	isolation_source	Compatibility
1.	ID: MT792532.1	Egypt	*Peziza vesiculosa*	fruiting body	99%
2.	ID: JF908568.1	Italy	*Peziza vesiculosa*	----------	99%
3.	ID: AF491626.1	USA	*Peziza vesiculosa*	----------	99%

The PCR technique and the analysis of nucleic acid amplification results have a great role in accurately diagnosing fungal species with high accuracy and distinguishing between species that have similar phenotypic characteristics. In which a research team led by Hansen *et al*., (2002) [8], determined the genetic diversity between species of the genus Peziza, and by using sequences from region (ITS) rDNA ITSI-5.8SITS2 for 83 samples for the purpose of determining the evolutionary relationships between species, including the fungus isolate *Peziza vesiculosa*.

Phylogenetic tree

After amplifying the ITS target gene, and conducting the sequence process, and inserting the obtained nucleotide sequences, the genetic tree that was drawn with a total length of branches estimated by 0.00788317. The tree was drawn within fixed scales with branch lengths in the same units. The evolutionary distances used to build the phylogenetic tree with local isolate.

The evolutionary distances were calculated using the Maximum Composite Likelihood Method, which is based on the process of changing bases of each site on the gene to be examined. The analysis included (3) isolates in addition to the local isolate and knowing the percentage of its genetic affinity. In addition, based on the results of analysis of the genetic evolutionary tree, which is divided into two main clusters. The first cluster included the most closely related species for the local isolate, which are the isolate with serial numbers in the Gen Bank, MT792532.1, JF908568.1, and AF491626.1, with a genetic match rate of 99% as shown in table (3).

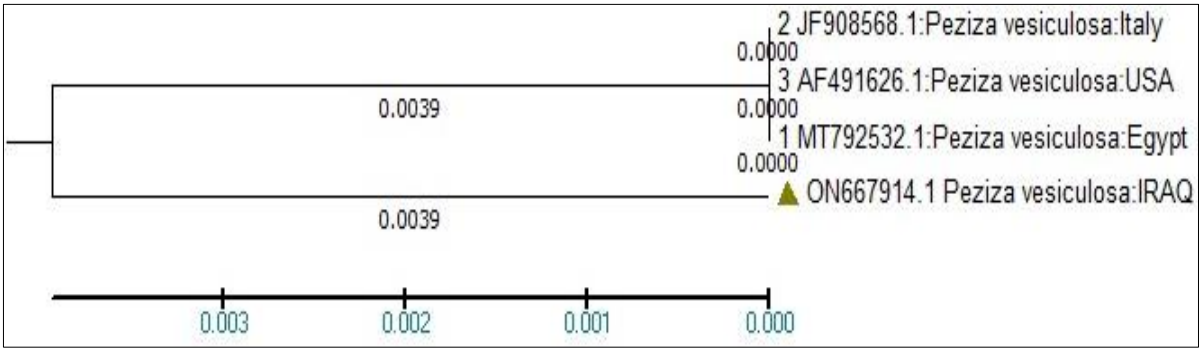

Fig 11: Genetic affinity tree for the local fungus *P. Versiculosa* and the global reference isolates.

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