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Morphological and Molecular Identification of New Record of Macro-fungi (Agaricales) in Mosul, Iraq

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ABSTRACT

Schizophyllum mushroom is considered a significant and crucial fungus that has great medical benefits. This mushroom is among the most significant species of fungus that cause wood to rot. In this study, it was feasible to obtain fruiting bodies for the *Schizophyllum* fungus on a trunk of a broken/cut Angodnia tree in Mosul city during March 2023. Through morphological and molecular identification and by the molecular methods of (PCR) technique, this mushroom was identified. The identification was based on the mushroom's Internal Transcript Spacer (ITS). Using these examination techniques, it was proved that the obtained fruiting bodies belong to *S. Radiatum* genus, which is regarded as the first recording in Iraq.

Keywords: ITS, Macrofungi, Phylogenetic tree, Agaricales, *Schizophyllum*, Microscope, and PCR.

INTRODUCTION:

Macrofungi are considered the richest and most diverse species in the world. Macrofungi term refers to all types of fungi that grow fruiting bodies which can be seen with human sight (Chang *et al.,* 2018; Lu *et al.,* 2020). Most macrofungi belong to Basidiomycota & Ascomycota phyla. The most prominent macrofungi is the *Schizophyllum*, which is classified within the Schizophyllaceae family, and Agaricales order within Basidiomycota (Kirk *et al.,* 2008; Vellinga, 2013). The term *Schizophyllum* is originally derived from Latin, the first part Schizo meaning division, and the second part Phyllum meaning platelet (Siqueira *et al.,* 2016). The most prominent morphological characteristics for this mushroom is that is it resembles coral reefs ripples or it is like a Chinese fan shape, its color in between white and gray containing villi on the surface, and it is located on an upper membrane covering the gills. These gills are split into two parts. The mushroom shrinks during the long dry periods in order to protect the spores, whereas it opens during humidity periods to release the spores (Piepenbring, 2015) *Schizophyllum* mushroom was first described in 1815, and later was first classified by Linder in 1933, and Cook in 1961 through studying all the unique characteristics of this mushroom, which include the morphological & molecular features of many samples of the mushroom from around the world (Guzman, 2003). The importance of this mushroom lies in the fact that it has high-quality proteins, polycarbohydrates, also abundant of the unsaturated fatty acids beside the secondary metabolites. This made the mushroom an important and vital part of the Macrofungi used in treating many health problems, such as cancer. The modern biotechnology concentrates on these Macrofungi and their potential applications with great interest and attention. *Schizophyllum* has recently received an increased and wide interest due to its benefits in the tumors treatment

(Shamtsyan *et al.,* 2010). The Researchers' objective is to diagnose in Iraq, the original isolate from *Schizophyllum radiatum* and the Arab nation, also in order to identify the categorized fungus isolate identification by performing the (PCR) technique, and registering the isolate in the Genbank in addition to drawing the genetic affinity tree.

MATERIALS AND METHODS:

Samples Collection

During a scientific field trip to the forest of Mosul, the second largest city, located in northern of Iraq on both sides of Tigris River. Mosul's moderate climate has helped the presence of a diverse growth of various types of fungi. On the morning of March 19, 2023, the fruiting body was obtained from a broken Angodina, as well as a broken Mulberry tree, in which the fruiting bodies were collected and packed in special bags. Then, they were brought to the lab where they were washed several times to remove the dust and dirt. The fruiting bodies were cut into small pieces, then sterilized by ethanol (70%) / 2 min. Moreover, they were washed with DW and dried with filter papers. Finally, small pieces were planted in PDA dishes & incubated at a temperature of 28 Co for seven days (Ariffin & Idris, 1992).

DNA extraction

The obtained fungal isolate was stimulated & activated, the DNA was extracted from the fungal hyphae of the colony after seven days by using extraction kit from the Korean company "Macrogen" & according to the company's instructions & standards.

Electrophoresis

In order to conduct the electrophoresis, the agarose gel was prepared in TBE, and consisted of mMTris 40 and mM boric acid 20, and 1M from EDTA. Then, it was put in the microwave until boiling, left to cool down at a temperature of 50- 60 Co. After that, the agarose gel was poured into tools container after placing the comb in order to make holes without bubbles. Then, the comb was lifted/ removed and the gel was placed in the storing tank to pour the prepared liquid in order to cover the gel. Moreover, the UL5 that was obtained from the sample was prepared by mixing them with UL7 that was obtained from the prepared liquid in the pits. This stage takes about 2-3 hours by using 70 (V/cm). Also, an amount of 50 (ug/ml) of the ethidium bromide dye was added for 30 minutes, and then the gel is transferred in order to dispose the water. Finally, by using the ultraviolet rays, the test was conducted.

Polymerase Chain Reaction (PCR)

The PCR technique was conducted to the DNA, after the extraction and purification by the frontal primer: (CAAACCCATGTCGAATTGAGAAG), and the rear primer: (CAAGTTTGCATACACTCTGGAATCT) and by using a group of Primers provided by the Korean company "Marogen" (Chog *et al.,* 2011).

DNA sequences

The samples were prepared to identify and specify the DNA sequencing of the Nitrogen bases. The DNA was amplified by using the reaction mixture with a final volume reaching to ul25, which consists of a DNA template with a volume of 5 microliter, ul12.5 of PCR MasterMix and ul1 for each primer. The volume was completed by distilled water. The thermal amplification was conducted as follows: The denaturation point was at ninety-five-degree temperature with three min., followed by 35 cycles at 95 Co for 45 seconds for coloring.

Also, 55 Co for 1 minute for the sake of lengthening. The last elongation is 72 Co at seven min. The samples are sent to the Korean company "Macrogen" to determine the DNA sequencing. Upon the arrival of the results, the DNA Sequence which utilized in the international website for biotechnology information via the link: (http://blast.ncbi.nlm.nih.-gov\blast.cgi) to categorize the isolates based on the Genbank sequence.

The phylogenetic tree

The specified sequences were contrasted to which sequences restored the nucleotide sequences databases in the NCBI Genbank. The distance matrix tree was built using neighbor joining approach (Nei & Saitou, 1987). The phylogenetic tree topology was constructed through boot analysis (X500) by using MEGA-6 program (Abdulhadi *et al.,* 2020; Tamura *et al.,* 2013).

RESULTS AND DISCUSSION:

Morphological Recognition of the fungus isolate

The fruiting mushroom body was found spontaneously and chance upon in nature, on a trunk of a broken/cut Angodnia tree. The fruiting body was able to obtain its nutritional necessities and needs from the tissues of the

plant due to the mushroom's ability to secrete organic disposing enzymes even after it was infected. The fruiting body that causes wood- rotting is characterized by its ability growth in an individual scattered way, or in the form of densely superimposed layers of 7 or more heads on the top of each other. This fruiting body grows from a single base on the pieces of the wood, and it grows on the origins of the hard living or dead trees as well. The fruiting body lacks a stem, and it is easy to be distinguished though its spherical head with a convex like- shape, rather think, its edge twisted inward, which is covered with small white, or pale brown layer. In addition, the fruiting body was not damaged or eaten, and it has a unique and distinctive smell. The grow period of this fruiting body extends from January to April, and then it dries up and eventually becomes a twig. Its gills are free, convergent, crowded, slightly wide, has a white color, and closes after the basidiospores exit. The pure fungal/ mushroom colony, that is grown on the laboratory culture medium PDA, is characterized by a bright white cottony color. When a part from the edge of the fungal culture is taken by the use of glass slide at the age of 7 days, and dyed with lactophenol blue, the fungal hyphae were seen as transparent and in the form of tubular structures divided by transverse septa containing the clamp bonds. The fertile layer consists of gill- plates that contain a layer of intertwined fungal hyphae which can vary according to the difference of length and density of other species, and also it contains crystal granules.

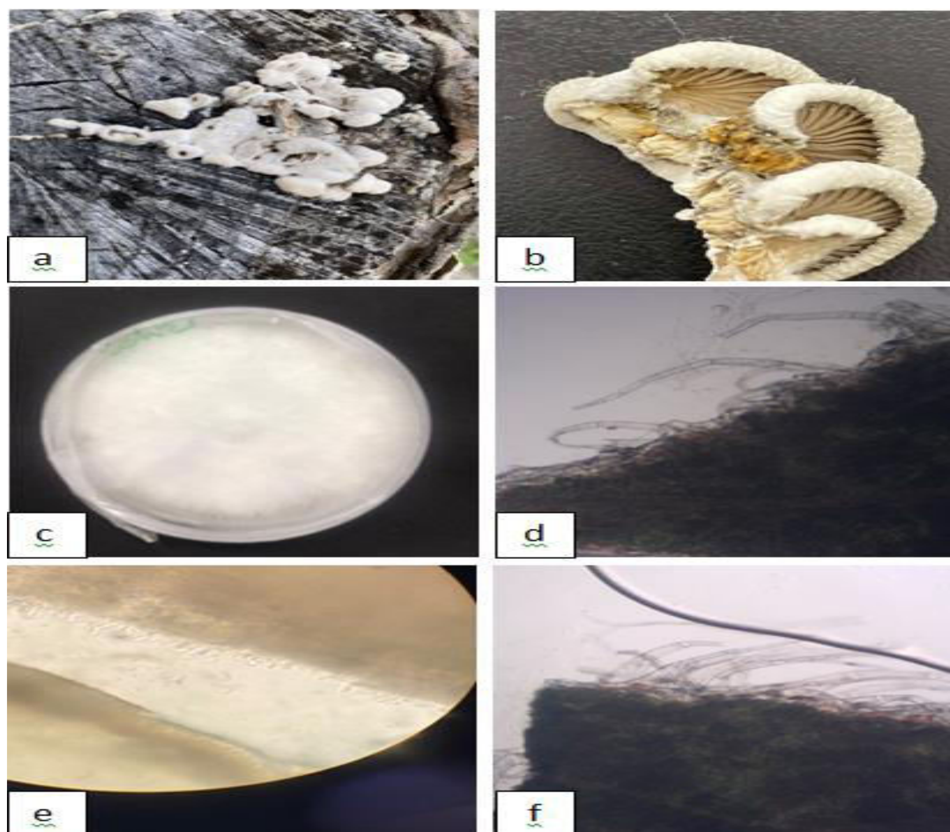

Fig. 1: A: Fruiting bodies, B: Gills, C: Fungal colony age 7 days, D, F: Clamp connection, E: Abhymenial hairs of *Schizophyllum radiatum*.

Molecular Identification of the fungal isolate

By depending on a pair of specialized primers and after DNA extraction from fungal hyphae of the selected fungal isolate, PCR method was used, in which it represents a simulation of what happens inside the living cell during the replication process. Such process helps to obtain many copies of the target gene by the numerical increase method. Also, by providing all the technical requirements with its three stages: (denaturation, binding, elongation), the outcome of the electrophoresis on the agarose gel, and the amplification of the target gene, showed a single horizontal

radiant bundle with clear and sharp ends. This result was shown after 35 cycles exposed under ultraviolet rays and after dyeing them with ethidium bromide in a dark room. The radiant bundle is of a molecular weight of 650 base pairs when compared with the bundles of the ready-made volumetric marker that is of a molecular weight of 1500 base pairs, which used in the first path of the gel paths. This was documented with many photos using digital camera to choose the best photo as show in figure (2). The appearance of one bundle shows how pure the extracted DNA is, how safe the primers and the chemical used are. In relation to this research study, literature and research referred to the recording of two new Basidiomycetes fungal isolates, and they are: *Schizophyllum commune* and *Schizophyllum radiatum* that were recorded in America and Belgium, by using PCR method. In order to amplify the gene, 4 DNA targets were amplified by using these pairs: 1ITS, 2ITS, 4ITS and 5ITS in another study conducted by (Chowdhary *et al.,* 2013).

Also, another research team led by (Singh *et al.,* 2013) two isolates were recorded *Schizophyllum fasciatum*, and *Schizophyllum umbrinum*, in Netherland, and Belgium by using PRC method, and the amplification of 4ITS, and 5ITS was used as well.

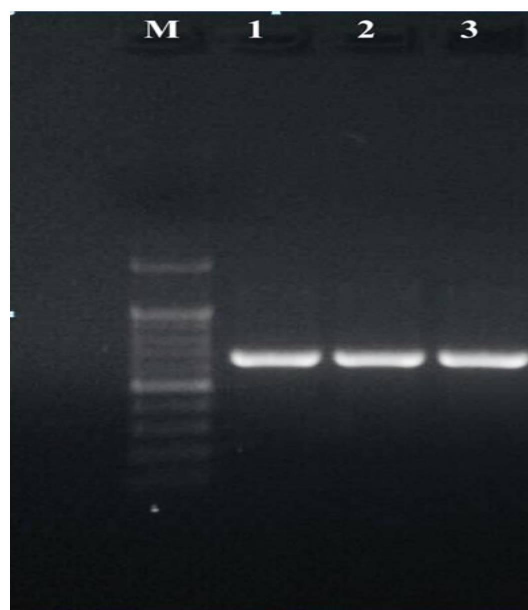

Fig. 2: DNA amplification products of the fungal isolated on agarose gel.

Nucleotide sequencing of the fungal isolate

After the completion of PCR By amplifying the required target using specialized extraction tools, the DNA bundle was cut from the agarose gel and placed into Eppendorf tube. Then, the frontal primer and deionized water were added to the tube. Finally, the results were sent to the Korean company "Macrogene" in order to identify the nucleotide sequences. The results have arrived after 30 days from the company via e-mail. The results carry several formats, including fasta (Gugnani, 2022).

Table 1: The serial number of the reference strain from USA, the percentage of its congruence with the selected local isolate *Schizophyllum radiatum* and the data of the variance with this isolate.

TTGACAGACCCTAATAAGTTAATACAACTTTCGACAACGGATCTCTTGGCTCTCGCATCG

Gene : 18S ribosomal RNA gene							
Identities	**Sequence ID with submission**	**Sequence ID with compare**	**Source**	**Nucleotide**	**Location**	**Type of substitution**	**No. Of sample**
99%	ID:OQ709247.1	ID: MH855328.1	*Schizophyllum radiatum*	A\T	179	Transvertion	1

Schizophyllum radiatum culture CBS:301.32 strain CBS 301.32 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: MH855328.1Length: 635Number of Matches: 1

Range 1: 45 to 635GenBankGraphicsNext Match Previous Match.

Score	Expect	Identities	Gaps	Strand
1062 bits(1177)	0.0	590/591(99%)	0/591(0%)	Plus/Plus

Query 1 TCAAACAAGTTCATCTTGTTCTGATCCTGTGCACCTTATGTAGTCCCAAAGCCTTCACGG 60
Sbjct 45 104
Query 61 GCGGCGGTTGACTACGTCTACCTCACACCTTAAAGTATGTTAACGAATGTAATCATGGTC 120
Sbjct 105 164
Query 121 TTGACAGACCCTAATAAGTTAATACAACTTTCGACAACGGATCTCTTGGCTCTCGCATCG 180
Sbjct 165 ....**A**.. 224
Query 181 ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA 240
Sbjct 225 284
Query 241 ATCTTTGAACGCACCTTGCGCCCTTCGGTATTCCGAGGGGCATGCCTGTTTGAGTGTCAT 300
Sbjct 285 344
Query 301 TAAATACCATCAACCCTCTTTTGACTTCGGTCTCGAGAGTGGCTTGGAAGTGGAGGTCTT 360
Sbjct 345 404
Query 361 GCTGGAGCCTAACGGATCCAGCTCCTCTTAAATGCATTAGCGGATTTCCCTTGCGGGATC 420
Sbjct 405 464
Query 421 GCGTCTCCGATGTGATAATTTCTACGTCGTTGACCATCTCGGGGCTGACCTAGTCAGTTT 480
Sbjct 465 524
Query 481 CAATAGGAGTCTGCTTCTAACCGTCTCTTGACTGAGACTAGCGACCTGTGCGCTAACTTT 540
Sbjct 525 584
Query 541 TGACTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAA 591
Sbjct 585 635

Fig. 3: The nucleotide sequences of the reference isolate confirmed in Gene bank with the sequence number.

MH855328.1 showing the sites of heterogeneity with the local isolates *Schizophyllum radiatum*. After obtaining these nucleotide sequences, they were deposited in the Genbank after 24 hours of arrival. This bank includes wide and comprehensive database that is available for free for everyone. In order to discover the identity of the fungal isolate, an Alignment process for the nucleotide was conducted by using Blast search, available in the website NCBI http://www.ncbi.nlm.nih.gov/blast The alignment process results showed that the nucleotide sequence for the selected local isolate which was deposited through copy and paste method, belong to the type *Schizophyllum radiatum*. As a result, there was a matching rate of 99% with the nucleotide sequences deposited submission with those reference sequence from China, 301.32 *Schizophyllum radiatum* strain CBS, that was registered under the serial reference number: 855328.1 Sequence ID:MH. And by clicking the serial reference number, a variation in the level of one nitrogenous base has appeared at the site 179, which replaced the nitrogenous base A with the nitrogenous base T.

The Phylogenetic tree

By using Mega program, version 6, that is available on the website NCBI, the genetic affinity tree was drawn. Based on the obtained nucleotide sequences for the local fungal isolate, and the nucleotide sequences for the isolates recorded and deposited in the Genbank, the genetic affinity tree was divided into two main clusters, as shown in **Fig. (4-6)**. The first cluster branched into secondary clusters, the closest to the local isolate were the isolates deposited in the Genbank with

the following serial reference numbers: 571060.1AY, 217537LT1. and 855328MH1. The matching rate was 100%. Whereas the second cluster included the Chinese isolate that is genetically farthest from the local isolate which carries the serial reference number: 050645.1KP with a matching rate of 99.98% The previous research papers proved that the PCR technique and the analysis of the DNA amplification results play a great role in diagnosing the various types of fungi with high accuracy, especially those isolates with the high degree of closeness or proximity. In addition to finding out many types of fungi species that have medical and industrial importance. In the same context (Yasuko *et al.,* 2016) pointed out to recording of a new fungal isolate for the first time in Brazil and America that belongs to *Schizophyllum umbirnum*, and based on their special primers in the region 4ITS, and 5ITS.

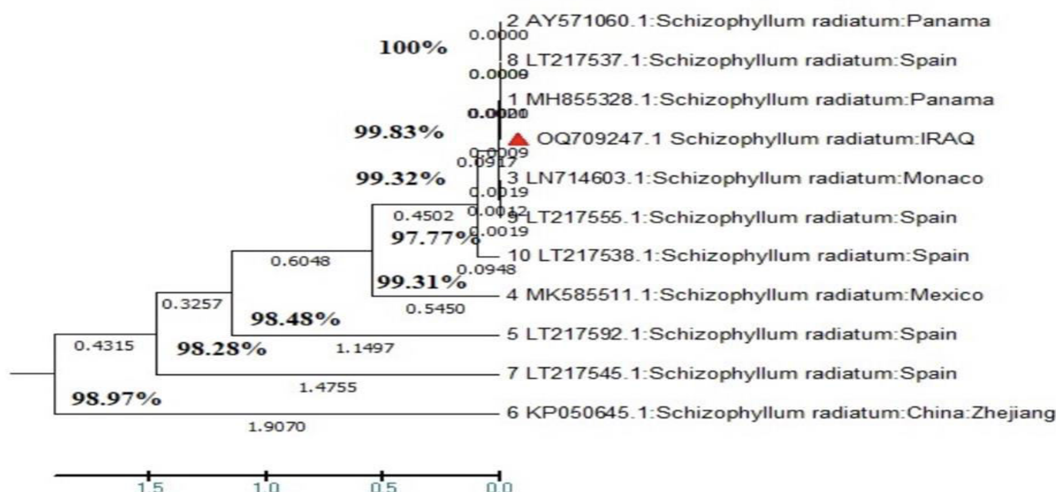

Fig. 4: Phylogenetic tree of the local isolate *Schizophyllum radiatum* and global isolates.

Table 2: Genetic similarity ratios for the local isolate *Schizophyllum radiatum* compared to the reference sequences global loaded in the NCBI.

Accession	Country	Source	Compatibility
ID: MH855328.1	Panama	*Schizophyllum radiatum*	99%
ID: AY571060.1	Panama	*Schizophyllum radiatum*	99%
ID: LN714603.1	Monaco	*Schizophyllum radiatum*	99%
ID: MK585511.1	Mexico	*Schizophyllum radiatum*	99%
ID: LT217592.1	Spain	*Schizophyllum radiatum*	98%
ID: KP050645.1	China: Zhejiang	*Schizophyllum radiatum*	99%
ID: LT217545.1	Spain	*Schizophyllum radiatum*	98%
ID: LT217537.1	Spain	*Schizophyllum radiatum*	99%
ID: LT217555.1	Spain	*Schizophyllum radiatum*	99%
ID: LT217538.1	Spain	*Schizophyllum radiatum*	97%

CONCLUSION:

There are a tremendous number of fungal isolates species that have the ability to grow or produce fruiting bodies in the city of Mosul. In this city, researchers can obtain new fungal isolates depending on the morphological and molecular methods which can identify and determine the isolates identity very accurately.

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CONFLICTS OF INTEREST:

The authors declare no potential conflict of interest.

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