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ORIGINAL ARTICLE

NEW RECORDS OF TWO MACROFUNGI SPECIES BASED ON MORPHOLOGICAL AND MOLECULAR IDENTIFICATION IN IRAQ

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ABSTRACT

This study was done in Al-Alam City, Salah Al-Din Province, to determine the diversity of the macrofungi in it. The results of the field study showed two species were recorded in Iraq for the first time, *Inocutis tamaricis* (Pat) Fiasson & Niemelä, 1984 (Basidiomycota, Hymenochaetales) and *Melanoleuca castaneofusca* Contu, 1998 (Basidiomycota, Agaricales). These species were diagnosed based on macroscopic and microscopic, DNA sequence analyses and environmental charactes. The study included the adoption of the *ITS* gene for molecular diagnosis, the results of which were confirmed for morphological and environmental diagnosis, and the specimens were registered in the NCBI Global GenBank under the international accession numbers OP153814.1 and MZ334407.1 for the species *I. tamaricis* and *M. castaneofusca*, respectively.

Keywords: Basidiomycota, *Inocutis tamaricis*, Iraq, Macrofungi, *Melanoleuca castaneofusca.*

INTRODUCTION

There has been great interest in mapping the macrofungi of the main geographical regions to obtain their distribution records similar to those of flowering plants (Meuller *et al*., 2007). However, unlike plants, measuring macrofungal diversity depends on the collection of fruiting bodies, which in turn depends to a large extent on the availability of moisture, that is, on seasonal rains, so it abounds in the spring and autumn due to the high humidity and abundance of plants (flora) at these times (Sibounnavong *et al*., 2008).

Iraq occupies a total area of 437,072 km2 (Al-Ansari, 2021) and is characterized by its different ecosystems and plant diversity. However, Iraqi macrofungi are overlooked and unexplored in many regions, despite their environmental and applied importance. There are very few and scattered studies in this regard, including the study of Aziz and Toma (2012), Toma *et al.* (2013), Al- Qaissi (2013), Al Anbagi (2014), Suliaman *et al*. (2017), Owaid *et al*. (2018), Al- Khesraji *et al*. (2019, 2020, 2022), Al Anbagi *et al*. (2021), Al- Khesraji *et al.* (2021), and Al Anbagi and Al- Khesraji (2022) reflecting an increase in the macrofungal species recorded with most of the documented species belonging to the phylum

Basidiomycota and the remaining species belonging to the phylum Ascomycota. That indicates the rich diversity of macrofungi in Iraq.

During the field trips to the Al-Alam area, the two species were collected and identified. Therefore, the present study is a new addition to the macrofungal record in Iraq.

MATERIAL AND METHODS

Collecting, identification and preserving macrofungi specimens: Macrofungi were collected from the orchards of Al-Alam City (34°38′41″N43°42′0″ /elevation 96 m), Salah Al-Din Province, in December-January 2022. Information about the habitat, habit, substrate, the host, and the nature of growth was recorded if it was solitary, overlapped, or clustered. Other information related to the date and place of collection, the color of the fruiting bodies with macrofungi in different parts, and the names of the plants prevalent in the area have also been documented.

The specimens were placed in plastic storage containers and transferred to the laboratory for macroscopic and microscopic examinations. Later, species were identified according to Ghobad-Nejhad and Kotiranta (2008), Sharma *et al*. (2013), Chinan *et al.* (2015), Kibby (2016), Sicoli and Mannarino (2017), and Antonín *et al*. (2021). Classification, synonyms, and basionyms were provided according to the GBIF Secretariat (2022). Some of the specimens were preserved in a preservation solution, ethanol alcohol 70%, with adhesive paper placed on the box. Information of the fungus, date and place of collection was written down, while the rest of the fruiting bodies were cut to small parts and dried on sterile paper by exposing them to indirect sunlight. The fruiting bodies were grinded by electric mill. The powder of the specimens was kept in a plastic storage container with a tight lid until use for genetic analysis. The identified fungi were deposited in the Department of Biology, College of Science, Tikrit University, Iraq.

Molecular studies: The DNA extraction was conducted using the MG Tissue Genomic DNA Extraction SV kit (Doctor protein INC, South Korea, Cat. no. MD014). The DNA amplification was completed using Dr. MAX DNA Polymerase (Doctor protein, cat. no.: DR00302). The primers for the amplification of the internal transcribed spacer region (ITS) were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990).

The PCR conditions were a denaturation at 95^{o} C for 5 min, followed by 35 cycles for a secondary denaturation at 95^{o} C for 30 sec; annealing at 55^{o} C for 30 sec; and an elongation at 72^{o} C for 1 min with a final extension step of 72^{o} C for 10 min. The PCR products were stored at 4^{o} C. Later, the PCR products were purified using Multiscreen filter plate, merck millipore. The amplified DNA was sequenced by Applied Biosystems ABI 3730XL DNA Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit, merck millipore Macrogen / Korea. The sequences were aligned using NCBI's Basic Local Alignment Search Tool (BLAST). The phylogenetic tree analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7 (Tamura *et al.*, 2013).

RESULT AND DISCUSSION

Morphological identification

In this study, two species belonging to the class Agaricomycetes were reported as new records for Iraq. Macroscopic and Microscopic descriptions and photographs were provided. The classification of them is as follow:

Kingdom: Fungi
Phylum: Basidiomycota
Class: Agaricomycetes
(1) Order: Hymenochaetales
Family: Hymenochaetaceae
Genus: *Inocutis* Fiasson & Niemelä, 1984
Speceis: *I. tamaricis* (Pat.) Fiasson & Niemelä (1984)
(2) Order: Agaricales
Family: Pluteaceae
Genus: *Melanoleuca* Patouillard, 1897
Species: *M. castaneofusca* Contu, 1998

Inocutis tamaricis (Pat.) Fiasson & Niemelä, 1984 (Pl. 1)
Basionym: *Xanthochrous tamaricis* Pat., 1984
Synonyms: *Inonotus tamaricis* (Pat.) Bondarzew & Singer
Inonotus tamaricis (Pat.) Maire
Inonotus tamaricis f. *corneus* Bondartseva
Polyporus tamaricis (Pat.) Sacc. & D.Sacc.
Xanthochrous rheades subsp. *tamaricis* (Pat.) Bourdot & Galzin

Basidiocarp: Sessile, hemispherical, rough texture (woody), 5.5-7.0 cm wide, 3-4 cm thick, creamy to rusty-brown with a lighter at the margin. Flesh: woody texture, dark brown at the center, mixed with pale yellowish and white mycelium. Hymenial layer: tubular, creamy to pale brown become dark brown at the edges ends with irregular pores. Basidia: 4-spored, hyaline in H_2O. Basidiospores: 5.5-7.5x5.0-6.0µ, oval to elliptical, yellowish to pale brown, thick-walled, smooth. Habit and habitat: solitary; fruiting on live and dead trees of *Tamarix* spp. Edibility: locally and globally unknown.

Distribution: Greece (Piatek, 2001), Southern Europe, Northern Africa, Southern Asia, China (Ryvarden, 2005), Iran (Ghobad-Nejhad and Kotiranta, 2008), India (Sharma *et al*., 2013), Romania (Chinan *et al*., 2015), Italy (Sicoli and Mannarino, 2017; Girometta *et al*., 2020).

Note: The current results were consistent with the aforementioned sources, which also confirmed that the presence of *I. tamaricis* on *Tamarix* trees is a distinctive feature in determining its identity.

Melanoleuca castaneofusca Contu, 1998 (Pl. 2)

Basidiocarp: Cap: 4.8-10.0 cm broad, smooth, depressed, slightly reflexed towards margin, laccate, dark gray to brown with a silver appearance, and darker spots around the cap margin, and entire cap margin. Flesh: white, fragile, the smell is similar to that of mushroom. Gills: white, subdeccurent, crowded, smooth. Stipe: 3.0-7.0x3.5-4.0 cm, white changed to brown

after harvest, central, equal, solid, fibrillose, longitudinally striate. Volva and ring and absent. Basidia: 4-spored, hyaline in H_2O, 12.5-15.0 x75.0µ. Basidiospore: 6.25-7.5 x 5.0-7.5µ, elliptical with central oil droplets and an ornamented wall. Spore print: is white to light yellow. Cheilocystidia: 35.0x7.5-10.0µ, lageniform with crystals at the apex. Pleurocystidia present and have a similar shape to cheilocystidia. Habit and habitat: solitary, collecting from the soil of barley fields. Edibility: locally unknown, globally edible (Singer, 1986).

Distribution: It has been collected from several countries such as Italy, Czech Republic, Slovakia, France, Britain, and Sweden (Kibby, 2016; Antonín *et al*., 2021).

Molecular identification and phylogenetic analysis

The analyzed portions of ITS rRNA sequencing for both presented species were between 660 and 689 base pairs. The blast search of the sequence similarities was identified the first and second species sequences as *M. castaneofusca* and *I. tamaricis*, respactively. The two identified sequences were submitted to the NCBI GenBank under the accession numbers OP153814.1 and MZ334407.1 for species, *I.tamaricis* and *M. castaneofusca*, respectively.

The pairwise sequence alignment of *I. tamaricis* appeared transversion and transition in the 152-158 and 282 nucleotide positions, respectively, when being compared with the refrance isolate with accession number GQ253453.1 form the Mediterranean Sea (Diag.1). On the other hand, the sequence alignments of *M. castaneofusca* exhibited transition at the 312 alignment position once the Iraqi isolates paired with Italyi isolates with the acession number MW491323.1(Diag.2) (Chinan *et al*., 2015; Zhuo *et al*., 2016; Wu *et al*., 2019).

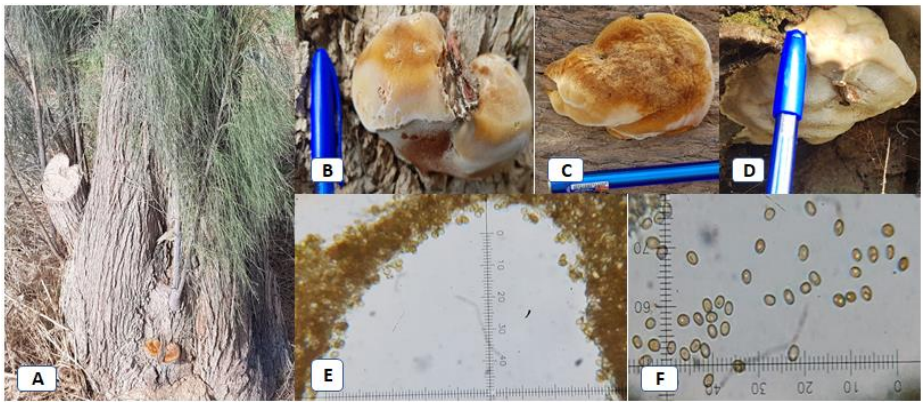

Plate (1): *I. tamaricis*; (A, B, C, D) Fruiting bodies on *Tamarix* tree, (E) Basidium and basidiospores, (F) Basidiospores. (40x).

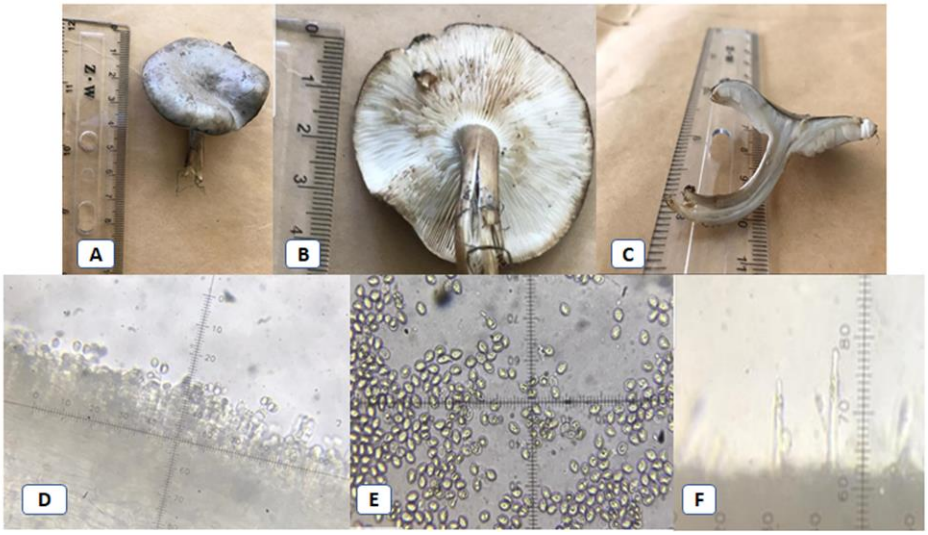

Plate (2): *M. castaneofusca*; (A) Fruiting body in lab, (B) Gills, (C) Long section of fruiting body, (D) Basidia and basidiospores, (E) Basidiospores, (F) cystidia. (40x).

Score	Expect	Identities	Gaps	Strand
1234 bits(1368)	0.0	687/689(99%)	0/689(0%)	Plus/Plus

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Query  1    AACGGTCTGCAGCTGGTGCGGGAACGCGCATGTGCTCGGCCTTTCGTGTTCAAATCCACT  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57   AACGGTCTGCAGCTGGTGCGGGAACGCGCATGTGCTCGGCCTTTCGTGTTCAAATCCACT  116
Query  61   CAACCCCTGTGCACCTTTGCGAAGCAAACAGTAGTAGTCGTCGTCTTTTCTTTTCTTTCT  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117  CAACCCCTGTGCACCTTTGCGAAGCAAACAGTAGTCGTCGTCGTCTTTTCTTTTCTTTCT  176
Query  121  GGTCGTGTGTTTTGAACCGCGGTCAAAAGTGAAAggggggAGAGGGCGGCGGTGAATGA  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  177  GGTCGTGTGTTTTGAACCGCGGTCAAAAGTGAAAGGGGGGGAGAGGGCGGCGGTGAATGA  236
Query  181  ATGCTTCGAGTTTTTCATTACAAACTACTTGTATGTCCTGTGGAACGTAATATGCTCCCT  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  237  ATGCTTCGAGTTTTTCATTACAAACTACTTGTATGTCCTGTGGAATGTAATATGCTCCCT  296
Query  241  CGTGGGCAAAATTGTAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAA  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  297  CGTGGGCAAAATTGTAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAA  356
Query  301  GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357  GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT  416
Query  361  TGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTTGAGTGTCATGTTAA  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  417  TGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTTGAGTGTCATGTTAA  476
Query  421  TCTCAAACCCTCAGTCTTTTGTTGACTCGAAGGACTGGGTCGGTTTGGACTTGGAGGTTT  480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  477  TCTCAAACCCTCAGTCTTTTGTTGACTCGAAGGACTGGGTCGGTTTGGACTTGGAGGTTT  536
Query  481  AACTGCTGGCTTTAGCAATTAGAGTCGGCTCCTCTTAAATTCATTAGCTGGACTTTGGTT  540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  537  AACTGCTGGCTTTAGCAATTAGAGTCGGCTCCTCTTAAATTCATTAGCTGGACTTTGGTT  596
Query  541  CGCATTTGCGGTGTAATAGTAACCAACTTGTTCGCCCGGGTGCTTGCCTAAAGAGTCTGC  600
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  597  CGCATTTGCGGTGTAATAGTAACCAACTTGTTCGCCCGGGTGCTTGCCTAAAGAGTCTGC  656
Query  601  TTCTAATCGCCTCCCAGTTGGGGCAAGTACTATATGACCCTTTGACCTCAAATCAGGTAG  660
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  657  TTCTAATCGCCTCCCAGTTGGGGCAAGTACTATATGACCCTTTGACCTCAAATCAGGTAG  716
Query  661  GACTACCCGCTGAACTTAAGCATATCATA  689
           ||||||||||||||||||||||||||||
Sbjct  717  GACTACCCGCTGAACTTAAGCATATCATA  745
```

Diagram (1): The pairwise sequence alignments of ITS region for both isolates *I. tamaricis* from Iraq (the query) and the Mediterranean Sea (the subject) with the accession number GQ253453.1. The alignment starts at the first base in the query sequence and progresses upwards to base 60 in the first alignment line; however, for the subject sequence, the alignment starts at base 57 and progresses downwards to base 745.

Score	Expect	Identities	Gaps	Strand
1187 bits(1315)	0.0	659/660(99%)	0/660(0%)	Plus/Plus

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Query  1    ACTCGGTGGGTTGTTGCTGGCTCCCAGGAGCATGTGCACACTTGCCATTGTTTCATTCTT  60
Sbjct  1    ............................................................  60
Query  61   TCTCCACCTGTGCACCTTTTGTAGGCTTGGATATCTCTCAAAGGAGATTGTATCATTATC  120
Sbjct  61   ............................................................  120
Query  121  ATCTCTCTTGGACTTAGGGATTGTTTAGAAAACTTTCCTTTGCATTTCCAGCCTATGTTT  180
Sbjct  121  ............................................................  180
Query  181  ATTATAACATATATATATACACCCCATTCGTATGTTTTAGAATGTTTATATTTGGCCTAT  240
Sbjct  181  ............................................................  240
Query  241  TACAGGCTTTAAAACTTATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGA  300
Sbjct  241  ............................................................  300
Query  301  AGAACGCAGCGGAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT  360
Sbjct  301  ..........A.................................................  360
Query  361  TTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAA  420
Sbjct  361  ............................................................  420
Query  421  TTCTCAATCCTTTCTGGGCTTATTCTCAGTTGGGCTTGGATATGGGGGACTGTTGCTGGC  480
Sbjct  421  ............................................................  480
Query  481  TTTGCAAAAAGTCAGCTCTCCTTAAAATTATTAGCAGGACATTTGTTGCAACCTTCTATC  540
Sbjct  481  ............................................................  540
Query  541  TGGTGTGATAGTTATCTACATCATAGATTATGTGCAGTTTATTATGTCTGGCTTCTAACA  600
Sbjct  541  ............................................................  600
Query  601  GTCCAATTAACTTGGACAACACTCTGATGATTTGACCTCAAATCAGGTAGGACTACCCGC  660
Sbjct  601  ............................................................  660
```

Diagram (2): The pairwise sequence alignments of ITS region for both isolates *M. castaneofusca* from Iraq (the query) and Italy (the subject) with the accession number GQ253453.1. The alignment starts at the first base in the query sequence and progresses upwards to base 60 in the first alignment line; also, for the subject sequence, the alignment starts at base 60 and progresses downwards to base 660.

The results of the ITS sequence region showed that the percentage similarities of the isolets *I. tamaricis* from Iraq were 99% with the Mediterranean Sea, Romania, and Greece, while they were matched as 98%, 97%, 96%, and 95% with South Korea, France, Italy, and China, respectively as shown in Table (1).

Table (1): Similarity ITS gene for the Iraqi isolate of *I. tamaricis* with Gene Bank isolates.

No.	Accession number	Country	Source	Similarity (%)
1	GQ253453.1	The Mediterranean Sea	*Inocutis tamaricis*	99
2	KJ755854.1	Romania	*I. tamaricis*	99
3	KX881614.1	Greece	*I. tamaricis*	99
4	AY558604.1	South Korea	*I. tamaricis*	98
5	MH855326.1	France	*I. tamaricis*	97
6	GU111920.1	Italy	*I. tamaricis*	96
7	HM050416.1	China	*I. tamaricis*	95
8	JN169789.1	China	*I. subdryophila*	89
9	KY907684.1	USA: Arizona	*I. jamaicensis*	83
10	MN498104.1	USA: Arizona	*I. dryophila*	94
11	MK422156.1	Tunisia	*Inonotus levis*	79

Further, the ITS sequencing results for Iraqi isolate of *M. castaneofusca* appeared the Similarity 99% with Italy , Czech Republic, Italy, Slovakia, and France , whereas compatible 98% with United Kingdom as in Table(2).

Table (2): Similarity ITS gene for the Iraqi isolate of *M. castaneofusca* with GenBank isolates.

No.	Accession number	Country	Source	Similarity (%)
1	MW491323.1	Italy	*Melanoleuca castaneofusca*	99
2	MW491321.1	United Kingdom	*M. castaneofusca*	98
3	MW491320.1		*M. castaneofusca*	99
4	MW491325.1	Czech Republic	*M. castaneofusca*	99
5	MW491324.1		*M. castaneofusca*	99
6	MW491322.1	Italy	*M. castaneofusca*	99

The results of the phylogenetic tree analysis of *I. tamaricis* showed that the current isolate was close to the Romanian isolate (Diag. 3). The p-distances between the previous species were 0.0013 and between the isolates from Iraq and the Mediterranean Sea were 0.0020. Moreover, variable distances 0.013-7.8 were observed between the Iraqi sequence and other GenBank sequences. The current results were comparable with other reported mean sequence divergences for fungi of 0.004-0.036 (Chinan *et al*., 2015) and 0.025 (Schoch *et al*., 2012).

The Iraqi isolate had a high bootstrap value (>79%) compared to other species in the GenBank isolates. Another study by Chinan *et al.* (2015) examined the phylogenetic evolution of *I. tamaricis* from Romania. The phylogenetic bootstrap value of the investigated species was high >65% compared with other sequences in GenBank.

The phylogenetic tree analysis was generated for the Iraqi collected specimen of *M. castaneofusca* (Diag. 4). The sequences of the Iraqi isolate were close to other sequences from Italy, France, and Slovakia. Their p-distances between them were 0.0008. The distances were varied from 0.0008 to 0.0025 between the sequences of the investigated Iraqi species and others in GenBank. The Iraqi isolate had a high bootstrap (>98%) with the isolates in GenBank. However, other studies showed two major clades: subgenera *Melanoleuca* (clade A) and subgenera *Urticocystis* (clade B) when phylogenetic analysis was conducted depending on the ITS region. These clades had high bootstraps of 99 and 98% respectively, (Kalmer *et al*., 2018). Another study conducted the phylogenetic analysis for other species of

Melanoleuca, including *M. angelesiana*, *M. castaneofusca*, *M. luteolosperma*, *M. pseudopaedida*, and *M. robertiana* and the bootstrap results was >75% (Antonín *et al*., 2021).

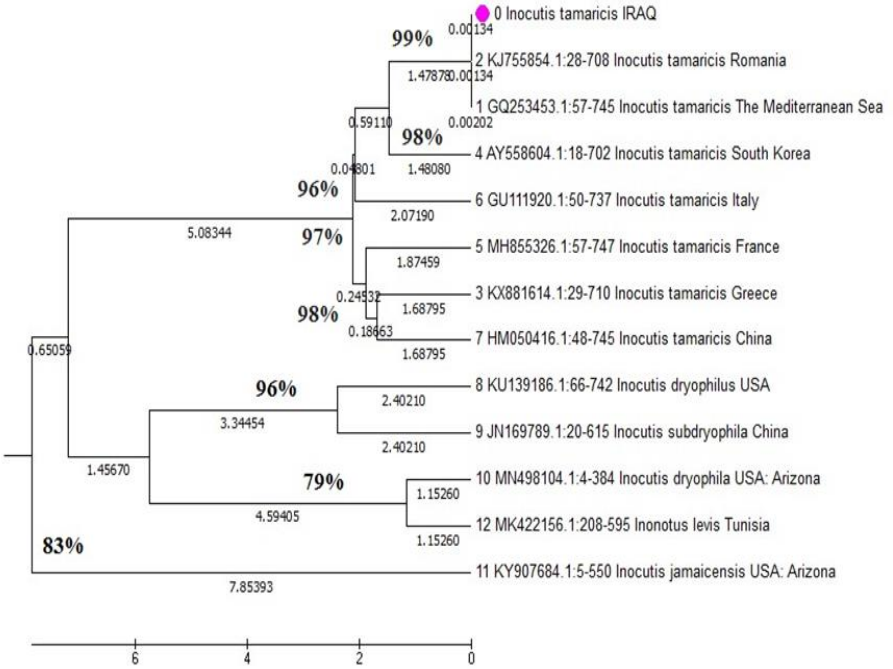

Diagram (3): Maximum likelihood tree depended on ITS sequence. Bootstrap values > 79% based on 1000 replications. (The sequence of *I. tamaricis* of Iraq is pink circle).

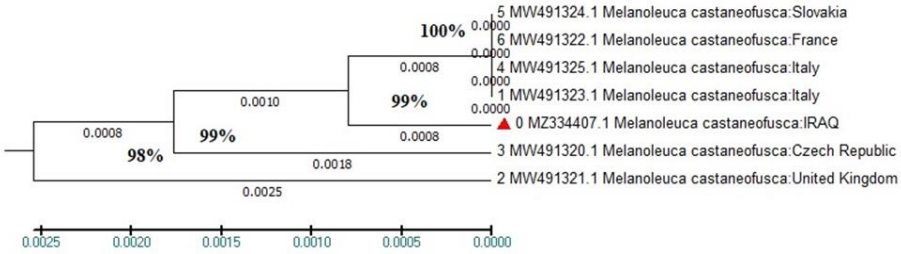

Diagram (4): Maximum likelihood tree depended on ITS sequence. Bootstrap values > 98% based on 1000 replications. The sequence of *M.castaneofusca* of Iraq is red triangle**.**

CONCLUSIONS

The present study reported the first *I. tamaricis* and *M. castaneofusca* collected from *Tamarix* tree soil, respectively. The study also indicates the possibility of the presence of several macrofungi that were not recorded in Iraq. The molecular analysis confirmed the phenotypic analysis. Also, the phylogenetic analysis of the Iraqi isolates for *I. tamaricis* and

M. castaneofusca were appeared close to the isolates in GenBank**.** This shows the evidence of compatibility between phenotypic analysis and phylogenetic analysis.

CONFLICT OF INTEREST STATEMENT

The authors whose names are listed immediately below certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

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تسجيل جديد في العراق لنوعين من الفطريات الكبيرة Macrofungi استنادا الى التشخيص المظهري والجزيئي

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الخلاصة

أجريت هذه الدراسة بمدينة العلم / محافظة صلاح الدين لتحديد تنوع الفطريات الكبيرة فيها. أظهرت نتائج الدراسة الميدانية تسجيل جديد للنوعين *Inocutis tamaricis* (Hymenochaetales, Basidiomycota)(Pat) Fiasson & Niemelä, 1984 و *Melanoleuca castaneofusca* Contu, 1998 (Basidiomycota, Agaricales) لأول مرة في العراق . اذ تم تشخيصهما استنادا الى الصفات المظهرية والجزيئية والبيئية. هذا وتضمن التشخيص الجزيئي اعتماد الجين ITS والذي جاءت نتائجه مؤكدة لنتائج كل من الفحوصات المظهرية والبيئية، فضلا عن تسجيل العينات في بنك الجينات العالمي NCBI ضمن الرقمين الدولية OP153814.1 و MZ334407.1 للنوعين *I.tamaricis* و *M.castaneofusca* على التوالي.