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New Records of Three Basidiomycetous Species from Iraq Using Phenotypic and Phylogenetic Analyses

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Abstract

Wild macrofungi have potential functions in ecosystems and sustainable developments through their roles in waste management, human health and societal upliftment. However, there is limited knowledge of wild macrofungi in Iraq. Three interesting collections within *Chlorophyllum, Mycenastrum* and *Ossicaulis* genera related to basidiomycetes were discovered in central and northeastern parts of Iraq in 2022 during a field investigation on wild macrofungi. Molecular identification and morphological characterization were carried out for these collections. The molecular phylogenetic and phenotypic analyses confirmed that isolates are related to three species within two families (Agaricaceae and Lyophyllaceae) belonging to the order Agaricales and class Agaricomycetes. Based on the current publications, these species were the first records from Iraq. *Chlorophyllum agaricoides*, *Mycenastrum corium* and *Ossicaulis salomii* sequences were submitted as the first sequences from Iraq into the international biological database GenBank. The molecular phylogenetic tree formed three independent lineages in each genus with high support. The introduced species can easily be separated from closely related species in an evolutionary framework. The study's results might aid in further investigations into the macrofungi in Iraq.

Keywords

Chlorophyllum agaricoides; macrofungi; molecular analysis; *Mycenastrum corium*; *Ossicaulis salomii*

Introduction

Fungi kingdom exhibits extensive morphological and phylogenetic diversity (1) with their ecological, metabolic and other functions playing vital roles in the ecosystem and human life (2-4). To date, the fungal diversity is uncertain, with several species estimated from 1.5 to 12 million. Nearby 150,000 fungal species have been isolated or collected, described, named and classified from that number. Globally, 1000 to 2000 species have been recorded yearly since the introduction of the DNA technique for species identification (5).

Due to the limited studies, the fungal sexual forms producing visible fruiting bodies known as macrofungi have been focused on since 2012. Few studies have reported species related to Ascomycetes in Iraq (6-7). However, the majority of the recorded species are related to Agaricomycetes (1,8).

These surveyed fungi have been predominantly reported from the north of Iraq. The nutrients, temperature, light and humidity are sufficient for fruiting body formation and time to be collected and recognized. The Iraqi climate and vegetation diversity reflect macrofungi's rich growth and diversity in its regions. Particularly, Erbil, Sulaymaniyah and Salah al-Din provinces located in northern Iraq have been informed by many studies, most of which belong to the Division Basidiomycota (4,6,9-13). This study aimed to introduce new records from Iraq continually discover interestingly and precisely identify Iraqi mycoflora.

Materials and Methods

Sample collection and morphological characterization:

The specimens were collected during field trips through visual searches at various locations of the Mirjaban city, Sulaymaniyah 35°49'50"N, 45°10'27"E and Diyala 33° 57' 59.0688'' N and 44° 54' 57.8484'' E, in May-June 2022. The collected specimens were photographed in their natural habitats, and the location, date, temperature, and type of plants prevailing in the collection area of the specimens and their habits were documented. The specimens were then transported to the laboratory to observe the macroscopic characteristics, including (size, colour, texture and shape). Then the microscopic features, spores and cystidea were observed after tissue sectioning. The spores were imaged through a compound light microscope. Subsequent samples were identified according to (14-21), classification, basionyms and synonyms are presented according to (22). Some samples, listed in the morphological identification section, were preserved after drying in the herbarium of the College of Science/Department of Life Sciences, Tikrit University. While some samples were washed, the dirt was removed and cultured on potato dextrose agar in preparation for molecular analyses.

DNA extraction, PCR amplification and sequencing :

The genomic DNA of the macrofungal isolates was extracted from 200-300mg of pure culture of each species using the Plant Genomic DNA Extraction Mini Kit by following the standard protocol (Favorgen, Taiwan). DNA samples were stored at -20 ºC for later use after checking DNA quantity and quality as described in (23). The internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) were amplified using universal primers ITS1 and ITS4 by PCR technique for species identification of basidiomycete (24). A mixture of twenty-five µL PCR reactions composed of twenty µL of master mix, one µL of each primer (ITS1 and ITS4), two µL H2O, and one µL template DNA. The thermal cycling of the primer pairs was started at 95 °C for 5 min, followed by forty cycles of three thermocycles (95 °C for 30 s, 54 °C for 50 s and 72 °C for 1 min as the denaturation, annealing and amplification steps respectively) and a final elongation step (72°C for 10 min), which adapted from (24). The PCR amplicons were visualized for quality checking on 1% agarose gel in TBE buffer and sized using the DNA Ladder (23). The DNA molecules in the gel were photographed with an ultraviolet transilluminator using a Gel-Doc image analysis system. The Macrogen Company (Seoul, South Korea) used Sanger sequencing to clean and sequenced the PCR products. Later, phylogenetic trees were constructed from received sequences after bioinformatic analyses.

Bioinformatics and phylogenetic analysis:

The received ab1 sequence files from the sequencing company were uploaded to the Geneious software V. 9.1.8 (Biomatters Ltd., Newark, New Jersey) and trimmed. Forward and reverse amplicon sequences of ITS were combined to get consensus sequences. Preliminary molecular identifications of the consensus sequences were performed pairwise, comparing obtained sequences to the global database using BLASTn queries in NCBI. To build phylogenetic trees, GenBank sequences of the closest related species to studied fungal species were obtained (Table 1). The sequences were aligned using MAFFT v7.309 with the default parameters (25). Using MrBayes V3.2.6 (26), a phylogenetic tree was inferred in Geneious version 9.1.8 (27) with default settings and a general time-reversible (GTR) model. The final tree was visualized in Geneious version 9.1.8. Consequently, the obtained fungus's ITS sequences were sent to GenBank with the accession numbers PP556338, PP556337 and PP556336 for *Chlorophyllum agaricoides, Mycenastrum corium and Ossicaulis salomii,* respectively.

Results

Molecular identification of macrofungi

The presented results confirmed morphological species identification related to three families linked to Agaricomycetes as described later. The molecular study was also confidently identified at the species level after being blasted fungal sequences against the international database. The molecular phylogenetic results were constructed, and three independent lineages in each genus were formed with high support (Fig. 1).

Morphological identification of macrofungi

1- *Chlorophyllum agaricoides* (Czern.) Vellinga

Eukaryota: Fungi.

Dikarya: Basidiomycota.

Sub-Division: Agaricomycotina

Class: Agaricomycetes

Subclass: Agaricomycetidae

Order Agaricales

(1) Suborder: Agaricineae

Family: Agaricaceae

Genus: *Chlorophyllum Massee* 1898

Species: *Chlorophyllum agaricoides* (Czern.) Vellinga 2002

Synonymy: *Endoptychum agaricoides* Czern. 1845

Secotium agaricoides (Czern.) Hollós 1902

Chlorophyllum agaricoides (Czern.) Vellinga 2002 (Fig. 2).

Basidiocarp: secotioid; Cap:16.0-40.0mm broad, 25.0 mm high, globose to puffball–like, white to cream with fibrous scales; Margin: does not break free from the stipe;

Table 1. GenBank numbers of derived sequences of basidiomycetous species used in the phylogenetic analyses.

Species names	GenBank numbers of ITS
Asterophora parasitica CBS683.82	AF357038.2
Calvatia gigantea voucher 428462	MZ354632.1
Chlorophyllum agaricoides voucher 16968	JF908776.1
Chlorophyllum agaricoides voucher MICH8133	MN161870.1
Chlorophyllum agaricoides voucher NSK 1014448	MT302578.1
Chlorophyllum arizonicum NY 809153	NR_171848.1
Chlorophyllum demangei voucher Z.W. Ge 3574	MG741964.1
Chlorophyllum hortense voucher PREM VDW799	MG741970.1
Chlorophyllum levantinum ACAM 2012-320	NR_169974.1
Chlorophyllum lusitanicum AH 45540	NR_158317.1
Chlorophyllum lusitanicum voucher AH46465	MH368354.1
Chlorophyllum molybdites voucher CUH AM704	MT181061.1
Chlorophyllum molybdites voucher MHHNU 30237	MK239232.1
Chlorophyllum neomastoideum voucher JBRI-M23-182	OR852555.1
Chlorophyllum palaeotropicum PREM 62142	NR_159759.1
Chlorophyllum pseudoglobosum CUH AM155	NR_137967.1
Disciseda hyalothrix voucher NSK1014099	MN151399.1
Mycenastrum corium voucher JLF7614	MT378214.1
Mycenastrum corium voucher MJ5467	DQ112628.1
Ossicaulis lachnopus voucher NSK 1017077	OQ216534.1
Ossicaulis lachnopus voucher PRM:537802	HE649960.1
Ossicaulis lachnopus voucher PRM:824708	HE649959.1
Ossicaulis lignatilis voucher MICH340333	OM985829.1
Ossicaulis lignatilis voucher NAMA 2017-245	MH979294.1
Ossicaulis lignatilis voucher PRM:889177	HE649953.1
Ossicaulis lignatilis voucher PRM:889513	HE649954.1
Ossicaulis salomii isolate EGDA-OS2	MW915607.1
Ossicaulis salomii JLS3421	MK650044.1
Ossicaulis salomii voucher Ghobad-Nejhad 4324	MT535738.1
Ossicaulis yunnanensis isolate IH26	KY411961.1
Ossicaulis yunnanensis isolate IJ152	KY411962.1

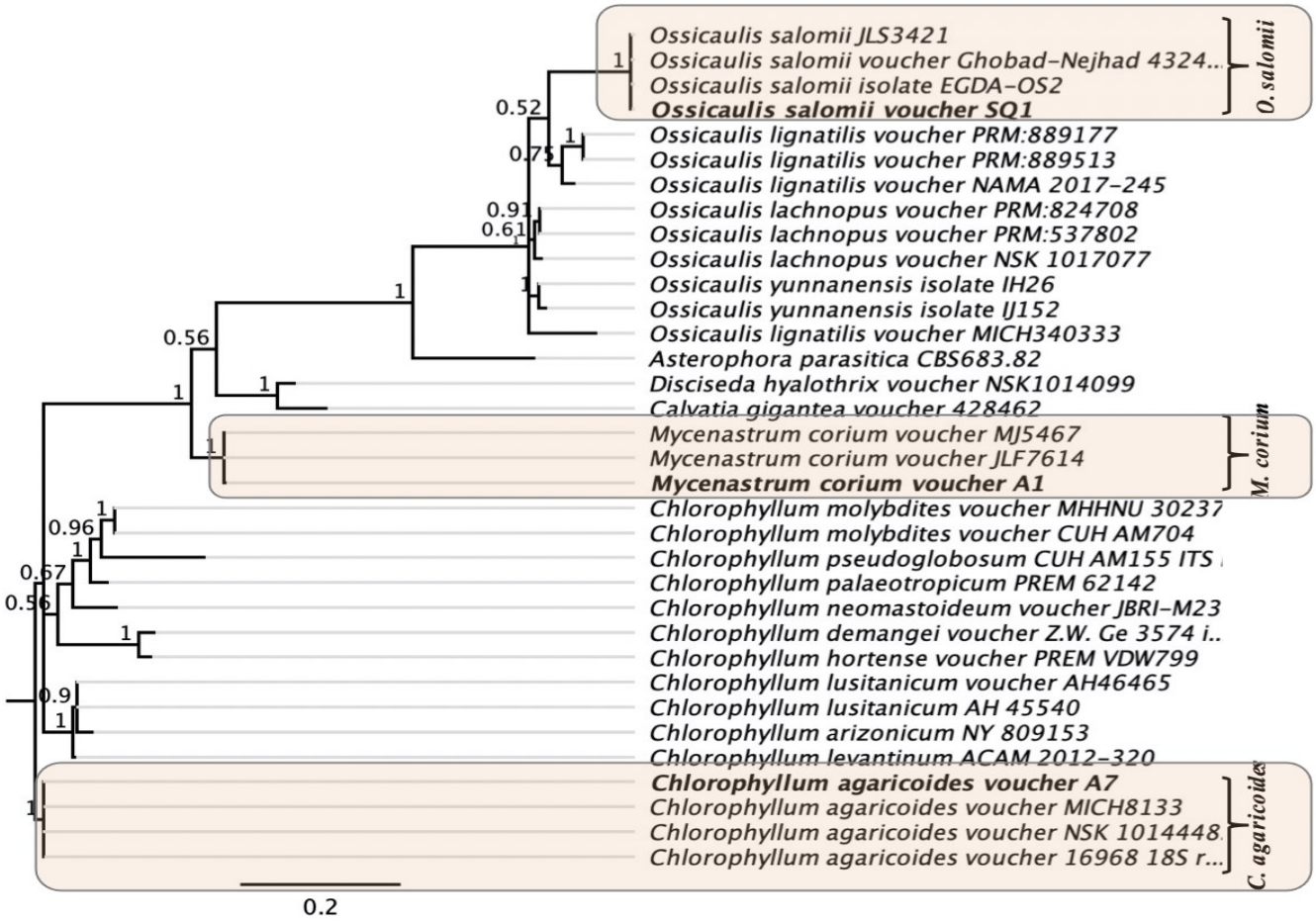

Fig.1. The ITS phylogeny tree of some Iraqi Agaricales species using Bayesian phylogenetic analysis. The nodes display Bayesian posterior probability values. Names of newly recorded taxa in this study were shown in bold

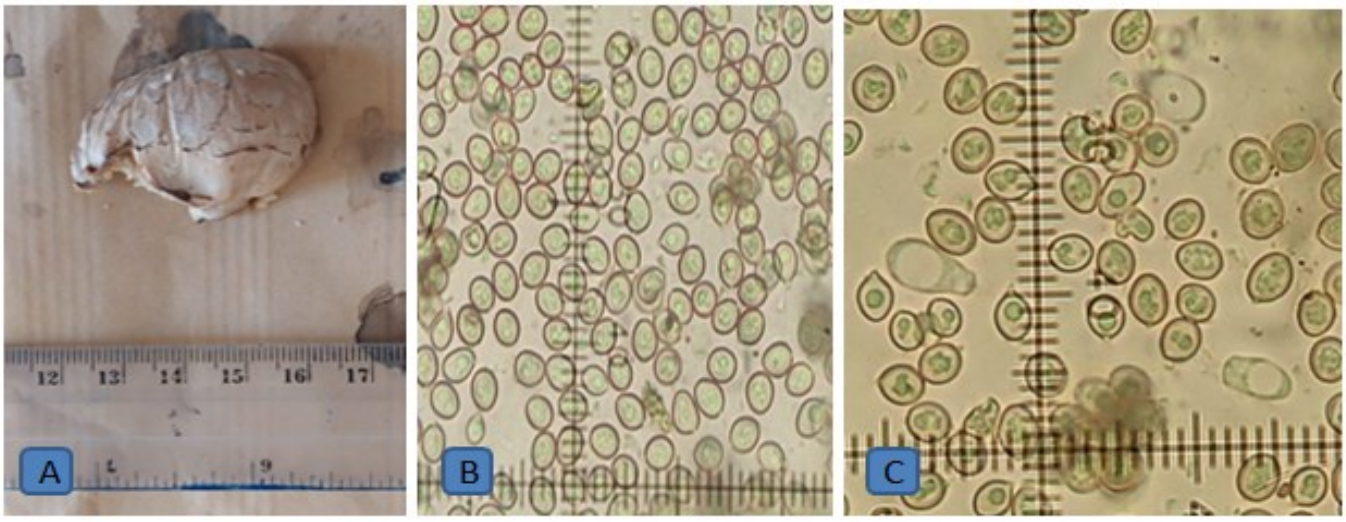

Fig. 2. *Chlorophyllum agaricoides*: a. basidiocarp, b-c. basidospores.(40x).

Hymenium layer: enclosed gleba, white, light brown at age; Stipe: 25.0mm long, 20.0mm wide, white to cream, internal (half of the stipe covered by the cap); Basidiospore: 7.5-10.0×5.0-7.5 µm, yellowish brown to green-brown, subglobose to ellipsoid, smooth with hailer appendage and most of it with central oil droplet; Edibility: locally unknown, globally edible; Habit and Habitat: solitary between grasses on mountainous areas dominated by *Hevea* spp. Trees land Distribution: China (14); Turkey (28-30); North America (19); Southeastern America (31); and Ukraine (32-33).

According to the taxonomic key, Bbasidiocarps of this fungus has sequestrate (secotioid), basidiospores from statismosporic, hymenophore (gleba) sub-lamellate to labyrinthiform, and the stipe from which pileus's margin does not break free (34). Based on the phylogenetic analysis and Bayesian tree, the isolate A7 of *C. agaricoides* located in the *C. agaricoides* clade includes other described vouchers of this fungus with high Bayesian Posterior Probability (BPP =1). The *Chlorophyllum* clade consists of the species *C. agaricoides* and other nine species. However, the *C. agaricoides* clade is separated from other *Chlorophyllum* (Fig. 1). This species has already been separated successfully from other closely related species using phylogenetic analysis. Based on a multigene phylogenetic tree, the genus *Chlorophyllum* consists of six sections: sect. *Endoptychorum* and sect. *Sphaerospororum*, sect. *Chlorophyllum*, sect. *Rhacodium*, Sect. *Ellipsoidospororum*, sect. *Parvispororum*. The fungus *C. agaricoides* belongs to a sect. *Endoptychorum* (34). The current results confirmed the ability of ITS sequences to identify and phylogenetically separate this species, and these results are aligned with previous results (20). The sequencing of the ITS region has been considered a suitable tool for species identification for the last 30 years due to its fastest evolving part of the rRNA cistron, high variation, and easy amplification (35). Besides, this region is substantially used as a DNA barcoding marker (3).

2- *Mycenastrum corium* (Guers.) Desv.

Genus: *Mycenastrum* Desv. 1842

Species: *Mycenastrum corium* (Guers.) Desv. 1842

Basionym: *Lycoperdon corium* Guers. 1805

Synonymy: *Endoneurum Czern*. 1845

Pachyderma Schulzer 1876

Mycenastrum corium (Guers.) Desv. (Fig. 3)

Basidiocarp: Sub-hypogenous, 74.0-85.0mm broad, globose, tapered to a pointed base; Exoperidium: White to creamy, smooth, cracked at age; Endoperidium: brownish, thick (2mm), tough, leathery; Gleba: White, turning to yellowish–green and pale brown at age, fleshy, firm; Subgleba: Absent. Basidiospore: 10.0- 12.5µm, bright–olive yellow, globose, thick-walled, spiny, reticulate, warted with hyaline pedicel and central droplet. Capillitium threads: 12.5 µm broad, thick, bright-olive yellow, branched with spiny; Edibility: Locally and globally unknown, Habit and Habitat: solitary, scattered, on dung. Distribution: Iran (36); Zimbabwe (37); Yemmen (38); Poland (39); Czech Republic (16); Turkey (20).

The current study identifies the species as *Mycenastrum corium* based on morphological description. The current isolation is the first report from Iraq. The Iraqi isolate resembles macro-micromorphological descriptions of *M. corium* samples collected from Turkey in 2019, but some differences exist. The Iraqi species has larger basidiocarps and spores than the Turkey isolate, characterized by a broad 50.0-60.0 mm basidiocarp and spores size 9.0-12.0 µm (20). Also, the Iraq isolates contain smaller spiny capillitium while the capillitium of the turkey isolates are 5.0-15.0 µm broad. The *Mycenastrum* Desv. Currently consists of only three gastroid species. These include *Mycenastrum catimbauense* Baseia, R.A.F. Gurgel, Melanda, R.J. Ferreira & Alfredo, *M. corium* (Guers.) Desv. and *Mycenastrum spinulosum* (Peck) Peck). *M. corium* is widely distributed and reported in Africa, Asia, Australia, Europe and North and South America.

Based on the results of the molecular analysis, the ITS gene sequence of the current collection has 100% base similarity with the reference sequences in the GenBank database. That sequence similarity was accurately identified as belonging to *M. corium*. The phylogenetic analyses also supported the identity relationship with *M. corium*, which had a high Bayesian posterior probability

Fig.3. *Mycenastrum corium:* a. basidiocarps, b-c capillitium. d-e basidiospores.(40x)

(BPP=1). The present species was independently parted from another genus, *Calvatia* and *Disciseda*, related to Agaricaceae and Asterophora, linked to Lyophyllaceae with high Bayesian posterior probabilities (BPP=1). The current results confirmed the ability of ITS sequences to reliably identify and phylogenetically separate this species, similar to previous results (20). The sequencing of the ITS region has been considered a suitable tool for species-level identification since the establishment of various databases dedicated to identifying ITS and other ribosomal sequences over the past 30 years (23). Besides, this region is substantially and constantly used as the official DNA barcoding marker for species identification of fungi (3,23). Thus, the conserved ITS region imparts substantial data for molecular phylogenetic studies as being applied in other genera (2,23).

To our knowledge, this study is the first description of the morphological characters and its molecular phylogeny using Bayesian phylogenetic analyses. Additionally, the species sequence from the Iraqi specimen is submitted into the worldwide GenBank database for the first time.

3- *Ossicaulis salomii* Siquier & Bellanger

Sub order: Tricholomatineae

Family: Lyophyllaceae

Genus: *Ossicaulis* Redhead & Ginns 1985

Species: *Ossicaulis salomii* Siquier & Bellanger, 2019.

Ossicaulis salomii Siquier & Bellanger (Fig. 4).

Basionym: Non

Synonyms: Non

Basidiocarp: Smooth, 9.0-50.0 mm broad to convex at first converting later applanate to slightly depressed at the centre, surface creamy when young hanging to brownish orange, inward folded edge sometimes slightly lobed which become beige, uplifted and crenulate at age; Lamellae: adnate-attached, slightly decurrent, crowded, white in young specimens, becoming creamy at age; Stipe: 9.0-19.0 mm x 1.0-7.0 mm, central to nearly central in young stages and being single or eccentric to lateral with age or once existence as a cluster, whitish to slightly light brownish with age, smooth, cylindrical, hollow, curved; Volva and Ring: absent. Basidia: 4-spored, hyaline in water; Basidiospore: Smooth 4.0-4.75 µm x 2.5-3.0µm, hyaline in water, sub-globose with hailer appendage. Habit and Habitat: Solitary, gregarious, on dead and wet plant remains and remnants of animal dung. Edibility: Unknown locally and globally. Distribution: The species was described, named and notified as new on 19 July 2019. *O. salaomii* was initially discovered in Spain Balearic Islands (40), and soon after, it was reported from Egypt and Iran (41-42).

Globally, the biodiversity of *Ossicaulis* species is relatively low and most of which have been recently discovered. Until now, seven species have been reported from this genus. The first two European species *O. lignatilis* and *O. lachnopus* were introduced initially in 1985 and 2007 respectively (44, 45). Afterward, *Ossicaulis yunnanensis* was described as a new species from China in 2018 and *Ossicaulis salomii* was reported in Spain in 2019 (40-43). Recently, three new species were identified as of 2023: *Ossicaulis* borealis and *Ossicaulis sichuanensis* were both discovered in China, *Ossicaulis semiocculta* was found in New Zealand (42). With inconsistent morphological traits and a lack of available molecular data, this genus does not have enough features to serve as the generic definition. However, the little phenotypic variables suggest these traits might be noteworthy at the

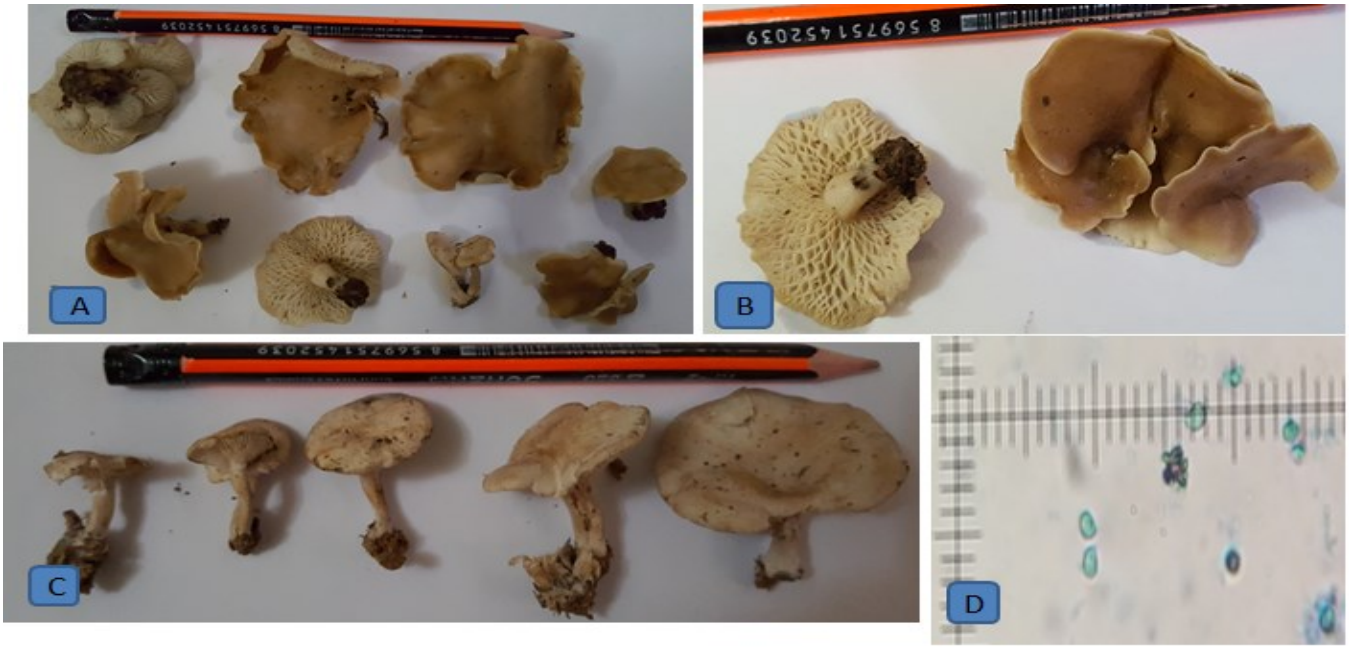

Fig. 4. *Ossicaulis salomii*: a-c. basidiocarps, d. basidiospores. (40x).

infrageneric level (40,43-44). Based on morphological techniques, the introduced species of this genus share various standard features except *O. salomiit*, making it difficult to distinguish the seven species as reported by previous studies (42-44). Thus, these species have been alienated based on the phenotypic and molecular phylogenetic results.

In the present results, the basidiocarp morphology and molecular data introduce *O. salomii* species as a new record from Iraq. According to the (17) taxonomic key of European species, the *Ossicaulis* genus is morphologically distinguished and molecularly separated into two species (*O. lachnopus* and *O. lignatilis*) depending on the size, especially lengths and widths of spores, as well as the color of the cap, the habitats of species and sequencing of DNA. Based on spore sizes and basidiocarp colours, *O. lachnopus* and *O. lignatilis* are used mainly to distinguish between the two species. Morphologically, the larger-spored collections (4.0-6.0 x 2.4-3.6 µm) and whitish to cream pileus represent *O. lignatilis*, while smaller spore size (2.8-4.0 x 1.6-2.8 µm) and grey or beige-grey tinged pileus characterize *O. lachnopus*. Also, together *O. yunnanensis* has faintly minor sizes of basidiospores (2.6-3.0×1.8–2.0µm) than those of *O. borealis* (3.0-4.0 x 2.0-2.4 µm) (43). Two more characteristics including the stem width of the basidiocarp and the size of the basidium are added to the taxonomic key of the five *Ossicaulis* species; *O. lachnopus*, *O. lignatilis*, *O. yunnanensis*, *O. borealis* and *O. salomii* provided by (21). The current Iraqi collections have a narrow stipe with a width of less than 20.0 mm and a length of up to 19.0 mm, similar to what is described as *O. salomii*. Nevertheless, the basidiocarps of Iraqi species are larger than those of *O. salomii*, which was first documented in Spain in 2019 (40).

Phylogenetically, the results of ITS region pointed the current agarical isolate SQ1 is related to the monophyletic group of *Ossicaulis salomii*, which is consistent with the phylogenetic result of *Ossicaulis* species (42). The Iraqi isolate clustered with the Egyptian isolate of *O. salomii*, revealing strong molecular separation (posterior probability=1) from other closely related species in the ITS phylogeny. The findings indicated that the introduced collection species clusters are a sister clade with solid support from *Ossicaulis lachnopus*, *O. lignatilis*, and *O. yunnanensis*. The Iraqi species is closer to *O. lignatilis* than to *O. lachnopus* and *O. yunnanensis* (40). In ITS tree, the current collection is closely related to the isolate of *O. salomii* EGDA-OS2 species. It is phylogenetically distinct from the isolate of *O. lignatilis* PRM:889177 (Fig. 1). The isolates of other species from the sequence database form an independently divergent clade to isolates of *O. salomii* and a sister to *O. lignatilis* with very statical support. Thus, the current Iraqi collections are molecularly closer to the Egyptian isolate of *O. salomii* and proposedly named *O. salomii*.

In this study, three new records species from Sulaymaniyah and Diyala provinces were morphologically described, genetically confirmed species identification and phylogenetically analyzed. The recently introduced species confirmed that the country has a highly diverse array of fungal species, as demonstrated in earlier publications' findings (46-47).

Conclusion

The current study recorded three new macrofungi species added to the Iraqi mycoflora list. In addition, the nucleotide sequences of these species were submitted to the international databases. This implies that ample research has been devoted to discovering a new species or records and investigating the distribution of these species in different areas of Iraq to detect macrofungal diversity, protection and sustainability. The study also suggested that the macrofungal diversity of Iraq might be underestimated.

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Authors' contributions

SQ conducted the study's morphological description and participated in the manuscript draft. RA and FR performed the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. AS was responsible for sample collection.

Compliance with ethical standards

Conflict of interest: The authors declare that there are no conflicts of interest regarding the publication of this article.

Ethical issues: None.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) did not use AI tools. The author(s) reviewed and edited the content as needed and took (s) full responsibility for the publication's content.

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