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Studying Phylogenetics Analysis for Macrofungi in Nineveh, Iraq

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A B S T R A C T

The study is focused on the phylogenetic relationships of macrofungi collected from Nineveh, Iraq, using a combined morphological and molecular approach. Generally, macrofungi are Fungi and play an indispensable role in ecological functions. They also have immense economic and medicinal value. The specimens were taken from different habitats: forests, grasslands, and agricultural soil. They were then identified morphologically based on both their macroscopic and microscopic features. Accurate species identification was performed by DNA sequencing of the internal transcribed spacer (ITS) region, and Maximum Likelihood was used for phylogenetic analysis.Seven fungal species have been identified, including four Basidiomycota: *Lentinula edodes, Schizophyllum commune*, *Psathyrella candolleana*, and *Candolleomyces candolleanus*; and two Ascomycota: *Diaporthe liquidambaris* and *Pochonia suchlasporia*. The morphology agreed with the molecular data, as the sequencing of ITS confirmed the species identification and showed evolutionary relationships. A phylogenetic tree demonstrated a distinct clade for each species, indicating genetic diversity within and among the tested taxa.

These findings allow pointing out the ecological importance of macrofungi in nutrient cycling, organic matter decomposition, species contribute to wood decay, and promising results of *Pochonia suchlasporia* in biocontrol. The medicinal properties developed by *Lentinula edodes* and *Schizophyllum commune* are believed useful in nutraceutical and pharmaceutical applications.The study represents a full overview of macrofungal diversity in Nineveh Province and thus indicates the relevance of classical taxonomy supplementation by molecular tools. The result will add to the global record of fungal biodiversity and point toward their potential use in medicine, agriculture, and biotechnology.

INTRODUCTION

Mushrooms are the popularly known as the "fructification" or "fruiting bodies" in macrofungi; they are indeed an important growth stage in their life cycle and are very common in the classes Ascomycota and Basidiomycota. These are fungi that are not only pivotal in ecosystem processes but also bring immense economic and nutritional value (toma *et al*., 2018). The term mushroom characteristically refers to the structures that at one point, under favorable environment, appear by growing in mycelium, variously shaped and colored depending on the species (Cho *et al*., 2020). The nutritional and therapeutic properties of mushrooms have been recognized for centuries. They are rich in bioactive compounds, including polysaccharides, terpenoids, phenolic compounds, and proteins. These compounds exhibit a wide range of bioactivities, such as antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, anticancer, and hepatoprotective properties (Sultan and Mula Abud, 2024; Mula abed *et al*., 2023). This group includes such well-known medicinal mushrooms as *Lentinula edodes* (Shiitake*), Ganoderma lucidum* (Reishi), and *Schizophyllum commune*. Traditionally, they have been applied in Eastern medicine for the treatment of a number of health disorders, ranging from inflammation up to cancer (Mizuno *et al*., 2013).

Among these, *Lentinula edodes* is recognized to be the most cultivated mushroom all over the world. It is commonly referred to as Shiitake. It is a saprophytic fungus growing on decaying wood. The mushroom is characterized by its flavorful sporophores, which are not only a popular food source but also a rich source of bioactive compounds (Puri, 2012). Its cultivation and consumption contribute significantly to the global mushroom industry, particularly in the production of nutraceuticals and functional foods (Kang *et al*., 2024).

Similarly, *Schizophyllum commune* is a notable macrofungus belonging to the Schizophyllaceae family. This species is very common on decaying wood, mainly in the rainy season. The organism being famous for its use in important metabolites-such as an immunomodulatory polysaccharide known as schizophyllan-tons of research work is carried out based on this for both medical and nutritional purposes. Besides, the fungus has also been proved to be a rich source of proteins, vitamins, lipids, and minerals and hence has been used as a dietary supplement in some culture (Hui-Min *et al*., 2016; Kumar *et al*., 2022).

Another interesting fungus family is that of Psathyrellaceae which includes the following species: *Psathyrella candolleana*. These dark-spored agarics are known for their fragile fruiting bodies and autodigesting properties, where mature fruiting bodies dissolve into an ink-like ooze. The taxonomy of this family has undergone significant revisions in recent years, with molecular sequencing techniques revealing distinct phylogenetic lineages. For instance, the Candolleomyces genus was identified as distinct from Psathyrella using genetic information and helped to further elucidate evolutionary relationships within this complex (Zhou *et al*., 2022). Alongside the Basidiomycota, the Ascomycota also contain ecologically and economically relevant fungi. For instance, members of the genus Diaporthe have been described both as plant pathogens and endophytes (AL-Ameri *et al.*, 2018). Members of this genus produce a wide variety of bioactive secondary metabolites, including alkaloids, terpenoids, and phenolics that have potential use in agriculture and medicine (Xu *et al*., 2021). Pochonia is a genus within the family Clavicipitaceae, comprising nematode parasitic fungi. These fungi are increasingly being harnessed in biological control strategies in the management of agricultural pests (Nonaka *et al*., 2013; Al-Tarjuman *et al.*, 2023; AL-Lashi, 2013). Fungal identification and classification traditionally are based on morphological features such as shape, color, and structure of the fruiting bodies and spores. Many of these methods have deficiencies in resolving ambiguities of taxonomy, especially in groups with cryptic species. Advances in molecular biology have revolutionized fungal taxonomy. DNA sequencing has emerged as one of the powerful tools for correct identification. For its high variability and easy amplification, the ITS region of ribosomal DNA is widely considered to be a "barcode" for fungal species (Bruns *et al*., 1991; Monocalvo *et al*., 2000; Yoo *et al*., 2022).

ITS-based phylogenetic analysis also showed the relatedness of fungi in relation to their divergence. Comparing ITS sequences between species thus enables investigators to construct a phylogenetic tree for the species representing genetic distance as well as ancestral lineages within and among fungal taxa. The

studies bear immense importance in providing descriptions of diversity and ecology within fungi, as well as their roles in biotechnological and medical aspects (Hibbett 1997).

The study of Macrofungal diversity in Iraq was done across the unique geographical region, diverse range of habitats available throughout the country-such as forest, farmlands, grasslands-support diverse fungal species; however, diversity was poorly documented about this area to date, considering molecular phylogenetic methods as well. So, this article tries to partially fill this lacuna by using two approaches-morphological and molecular-one for identification, and the analysis of macrofungi collected at different sites using, for the very first time in Nineveh,. It also attempts to unite the classical taxonomy approach with DNA-based techniques in hopes of a more profound understanding of the phylogenetic relationship among the fungi.

The present study involves the collection of fruiting bodies of macrofungi from natural habitats including Mosul Forest and surrounding agricultural areas in Nineveh. Taxonomic keys have been employed for identification at the morphological level, followed by molecular analysis of the ITS region for establishing the species with accuracy. Phylogenetic relationships among the identified species have been deduced using maximum likelihood methods, which may provide valuable information on their evolutionary course.

The wider implications of this study involve value for science and society in general. Ecologically, fungi are major contributors to nutrient cycling and ecosystem stability. Knowledge about their diversity and phylogeny will provide background information on conservation and sustainable land management practices. In an applied sense, the bioactive compounds they produce will have possible applications in agriculture, medicine, and in biotechnology. In contributing to the global effort of cataloging and conserving fungal biodiversity by documenting the macrofungal diversity of Nineveh, this study epitomizes untapped potential from organisms for human benefits.

METHODS

Sample Collection

Collection of the fruiting bodies of macrofungi was made from various localities in Nineveh Governorate: Mosul Forests, Al-Rashidia, Al-Gubba, Al-Fadhilia farms, and other agricultural areas. Collection started on 14th March 2024. The removal of the fruit body was done very carefully, either with sterile scalpels or even by hand, taking photos with indicative markers such as rulers or pens showing the size. Collection bags were labeled with vital information regarding the location of the site, habitat, and date of collection.

Preparation of Sample

Samples were washed with distilled water so as to wash off the adhering soil and debris. Specimen were then fixed in Formaline Acetic Acide (FAA) solution so that morphological features are fixed. Some of them are treated at 40°c in electric oven so to get them dry, then keep under amber coloured bottle. Others are fresh for culturing (Johnsy *et al.,* 2011).

Culture and preservation

Isolation of fungal colonies was done by surface-sterilizing of fresh fruiting bodies sections in a 2% solution of sodium hypochlorite for two minutes, followed by careful washing in sterile distilled water. Small pieces of tissue from the cap, stipe, or gills were subsequently placed on potato dextrose agar (PDA) - agar plates, incubated at a temperature of 28±2°C for 7-14 days, after which pure fungal colonies developed. These were sub-cultured into fresh PDA plates (kala *et al.* , 2020).

Isolates were maintained on PDA slants by topping it with 60% sterile glycerol for their long-term preservation and stored them at 4°C to be preserved for further molecular and morphological analysis (Valls *et al.,* 1999).

Morphological Identification

Identification of fungi was done based on the macroscopic features, including color, size, and cap and gill attachment, among others, but more specifically, their microscopic features concerning spore shapes, sizes, and ornamentation. Slides will be prepared using lactophenol cotton blue dye and will be observed under light microscopy for proper documentation of vegetative and reproductive structures (Nithya and Ambikapathy, 2014; Kuo, 2020).

DNA Extraction and PCR Amplification

Genomic DNA from mycelial cultures was extracted by the Thermo Fisher Fungal Genomic DNA Extraction Kit. The DNA concentration and purity were checked

on a spectrophotometer and gel electrophoresis, respectively. ITS amplification was made by PCR with the following primers: ITS1 and ITS4: Table (1).

Table 1. Show ITS amplification was made by PCR with the following primers: ITS1 and ITS4.

Forward primer	ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')
Reverse primer	ITS4 (5'-TCCTCCGCTTATTGATATGC-3')

The amplification products (~ 600 bp) were analyzed on agarose gel electrophoresis and viewed under UV light.

Phylogenetic Analysis

The obtained sequences of PCR were then compared with the reference sequences available in the NCBI database by BLAST. In the MEGA X software (Kumar S. *et al*., 2018), the Maximum Likelihood method/Kimura 2-parameter model was used to construct the phylogenetic trees (Kimura, 1980). To understand the reliability of the tree, Bootstrap analysis for 1,000 replicates was performed.

Thus, a combined approach yielded not only accurate identification but also insight into the evolutionary relationship of macrofungi in Nineveh.

RESULTS AND DISCUSSION

Identified Fungi

In total, seven fungal species were identified, of which four Basidiomycota: *Lentinula edodes, Schizophyllum commune*, *Psathyrella candolleana*, and *Candolleomyces candolleanus*, two Ascomycota: *Diaporthe liquidambaris* and *Pochonia suchlasporia*. These species have been found across diverse habitats such as decaying wood, grasslands, and agricultural soils, thus showing ecological adaptability for macrofungi in Nineveh (Table 2).

Table 2. Showing isolated fungi, their phyla, orders, families and habitats.

Fungal Species	Phylum	Order	Family	Habitat
Lentinula edodes	Basidiomycota	Agaricales	Omphalotaceae	Dead wood
Schizophyllum commune	Basidiomycota	Agaricales	Schizophyllaceae	Dead wood
Psathyrella candolleana	Basidiomycota	Agaricales	Psathyrellaceae	Grassland
Candolleomyces candolleanus	Basidiomycota	Agaricales	Psathyrellaceae	Humus-rich soil
Diaporthe liquidambaris	Ascomycota	Diaporthales	Diaporthaceae	Plant debris
Pochonia suchlasporia	Ascomycota	Hypocreales	Clavicipitaceae	Soil

Taxonomic features, both morphological and microscopic, were examined. *Lentinula edodes* had reddish-brown caps with white specks and creamy white mycelium, which is in agreement with the description of with Hamed (2022) and Mizuno *et al*., (2013). *Schizophyllum commune* had fan-like caps that were covered with dense soft hairs and longitudinally divided gills This agreement with Al-obaidy (2023). *Psathyrella candolleana* and *Candolleomyces candolleanus* were characterized by fragile fruiting bodies and spore morphology in accordance with the description of Zhou *et al*., (2022) and Nayana *et al*., (2023).

The woolly colonies were formed by Ascomycetes such as *Diaporthe liquidambaris* while *Pochonia suchlasporia* grew rapidly in yellowish colonies on PDA medium(Adhikari, Kim *et al*., 2016; Yang, Wang *et al.,* 2022) Fig. (1, 2).

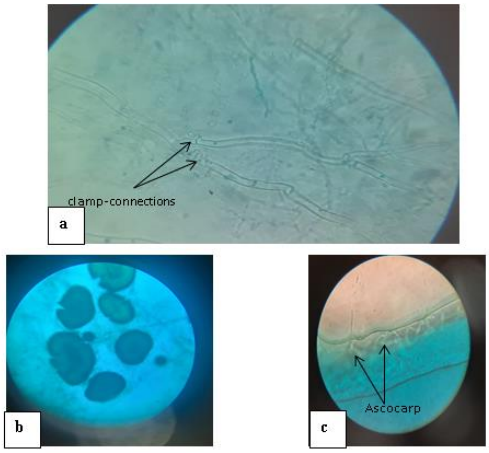

Fig. 1. a- clamp -connections in Basidiomycetes at 40x . b- Asexual shap in ascomycetes at 10X. c- Ascocarp in Ascomycetes at 40x.

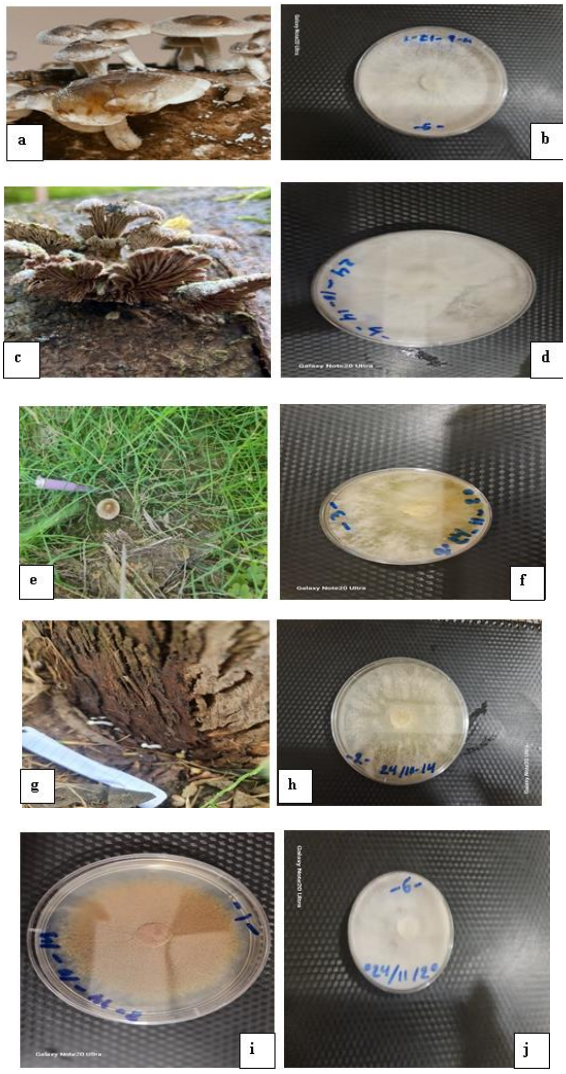

Fig. 2. a- fruiting body in habitat. and b-.mycelium on PDA. of L. edodes fungus. c- fruiting body in habitat .and d - mycelium on PDA . of Schizophyllum commune fungus. e- fruiting body in habitat and. f-. mycelium on PDA at 40x of Psathyrella candolleana fungus. g- fruiting body in habitat and h- mycelium on PDA . of Candolleomyces candolleanus fungus. i- mycelium on PDA of Diaporthe liquidambaris fungus. j-. mycelium on PDA. of Pochonia suchlasporia fungus.

Molecular Analysis

Successful DNA extraction was confirmed for all samples by PCR amplification of the ITS region, showing distinct single bands at ~ 600 bp. Species identification by sequence alignment and BLAST analysis was highly confident, hence reflecting the reliability of ITS markers for fungal identification (Harnelly *etal*., 2022), (Fig. 3.).

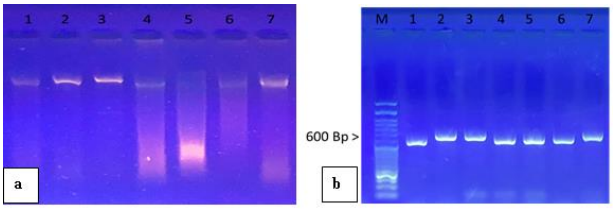

Fig. 3. a- showing genemic DNA from seven fungal sample b-showing PCR amplification of the ITS region(~600) from seven fungal sample.

Phylogenetic analysis further supported the morphological results, with a clear distinction between clades of each species shown in (Fig. 4).

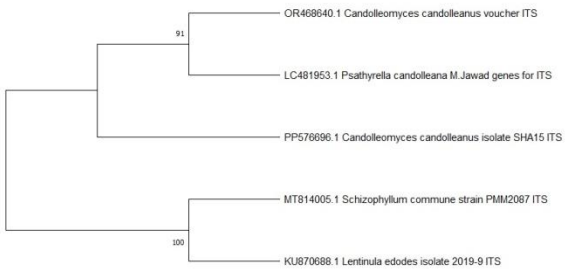

Fig. 4. Showing phylogenetic tree for five fungal sample.

Phylogenetic Relationships

A constructed phylogenetic tree using MEGA X software indicated a clear pattern of clustering. The close clades of *Candolleomyces candolleanus* and *Psathyrella candolleana* from the family Psathyrellaceae proved the closeness of the species phylogenetically. *Schizophyllum commune* and *Lentinula edodes* emerged in separate, distinct clades marking their divergence from the rest of Basidiomycota. Species of Ascomycota, such as *Diaporthe liquidambaris* and *Pochonia suchlasporia*

have created branches apart, representing their independent evolution (Fig. 4.).

Ecological and Biotechnological Implications

The diversity of the macrofungi identified here underlines their ecological importance in nutrient cycling and organic matter decomposition (Tran *et al*., 2018). Species such as *Schizophyllum commune* contributes to the decaying of wood, while *Pochonia suchlasporia* has the potential for application as a biocontrol agent against nematodes. Medicinal properties of *Lentinula edodes* and *Schizophyllum commune* further establish their biotechnological importance, whose applications range from nutraceuticals to pharmaceuticals (Mizuno *et al*., 2013).

CONCLUSION

This study identified and analyzed seven species of macrofungi from Nineveh, Iraq are (*Lentinula edodes*, *Schizophyllum commune*, *Psathyrella candolleana*, *Candolleomyces candolleanus*, *Diaporthe liquidambaris*, *Pochonia suchlasporia)*, by combining morphological and molecular approaches. Inclusion of ITS-based phylogenetics provided solid data on the evolutionary relationships between such organisms, contributing to the knowledge of fungal diversity and ecology within the region. This review evidences the importance of these fungi in medicine, agriculture, and even biotechnology and calls for the need for continued surveying and conservation of fungal biodiversity.

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Conflict of interests

The authors declare that there is no competing interest.

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