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Morphological and Molecular Characterization of *Pleurotus pulmonarius*

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Pleurotus pulmonarius, a nutritionally rich edible mushroom, has gained considerable attention for its adaptability, rapid growth, and medicinal properties. This piece of research is aimed to assess both morphological and molecular characteristics of *P. pulmonarius* to ensure precise taxonomic identification and to establish a reference for further research and strain development.Morphological characterization was carried out using freshly collected fruiting bodies grown under controlled environmental conditions. Macroscopic features such as pileus diameter, shape, color, stipe length, gill attachment, and spore print were carefully recorded. The pileus was observed to be convex to slightly oyster shell-shaped with a smooth, pale gray to white surface, while the stipe was lateral, fibrous and cylindrical. The gills were decurrent, closely spaced, and white to light cream in color. Spore prints appeared white. For molecular analysis, genomic DNA was extracted from pure mycelial cultures. The Internal Transcribed Spacer (ITS) region of ribosomal DNA was amplified using universal primers ITS1 and ITS4. PCR products were sequenced and compared with existing sequences in the NCBI GenBank database through BLAST analysis. The results confirmed a high sequence similarity (100%) with MN244439.2strain of *P. pulmonarius*, validating the morphological identification. The integration of morphological and molecular approaches provided a comprehensive characterization of *Pleurotus pulmonarius*, ensuring accurate species identification.

Keywords : Gills,ITS,pileus,*Pleurotus pulmonarius*, *r*DNA, stipe

INTRODUCTION

The term 'mushroom' is thought to originate from the French word 'mousseron' and generally comprised ofagaricoid fungi, classified under the order Agaricales of the class Agaricomycetes (Kirk *et al.* 2008; Kumar *et al.* 2022).Since ancient times, mushrooms have been regarded worldwide as a luxurious, gourmet food, and reliable nutritional source for their unique taste and delicate aroma. They are considered as a valuable source of essential nutrients such as dietary fiber, minerals and especially vitamin D.

Besides their nutritional value, mushrooms are also recognized as functional foods due to the presence of bioactive compounds that provide significant health benefits (Ren *et al.,* 2012). The chemically defined molecules extracted from *Pleurotus* spp. may hold great promise for their potential use as functional foods and as a source for developing novel and innovative drugs (Correa *et al.*2016). Mushrooms present a valuable alternative option to costly food items, which are also offering rich nutritional content. Moreover, because mushrooms can be cultivated using low-cost materials, they are easily cultivated by local farmers. As a result, mushroom cultivation is encouraged as an important step towards improving health and considered significant in sustainable development (Magolama *et al.* 2020).

The genus *Pleurotus*, commonly known as oyster mushrooms,a group of edible fungi that are widely cultivated across the globe,are highly valued for their nutritional, health, and environmental benefits. Nutritionally, their fruiting bodies are rich in protein and contain a wide range of essential minerals and vitamins. From a health perspective, they are regarded as wholesome

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and balanced food, often recommended for managing various health conditions. Environmentally, oyster mushrooms are typically grown on different substrates made from agricultural by-products, such as crop residues, which helps recycle organic waste and enhances its economic usefulness (Hassan and Ibrahim, 2022).

Pleurotus pulmonarius stands out among these, due to its vigorous growth, adaptability to grow on various agro-waste substrates, and advantageous agronomic traits. Precise identification and characterization of *P. pulmonarius*, are essential for mushroom breeding, conservation strategies, and assuring strain purity in commercial mushroom cultivation.Morphological characteristics such as cap shape, size, color, gill attachment, and mycelial growth have been used to identify and classify *Pleurotus* species. However, these features can often be affected by environmental conditions and may not provide sufficient information to differentiate closely related species or strains. Therefore, integrating molecular technology with classical taxonomy has become important for exact characterization of mushroom species.

Due to impact of global warming and the rising demand for protein-rich food to provide nutrition to the growing global population, the cultivation and commercialization of wild mushrooms have become a priority in rural agriculture and the food industry. Revolution in modern biotechnology has changed various facets of mushroom science. These facets include germplasm preservation mushroom cultivation technologies, the development of high-yielding and improved strains of mushrooms with enhanced biological efficiency, improved flavor and aroma, high nutritional content, and extended shelf life. Molecular biology plays a crucial role in various applications of the genetic enhancement and identification of cultivated mushroom species. Additionally, biotechnological innovations in mushroom science such as protoplast fusion, lignocellulosic degradation, and bioremediation are some other novel pathways for future development (Singh *et al.*2009; Miriyagalla *et al.* 2022).

Modern molecular techniques help in reducing the limitations of traditional classification systems, which often struggle with the inconspicuous characteristics, morphology uncertainty, and difficulty in discrimination between closely related fungal species (Blackwell *et al.,* 2006; Nilsson *et al.*2011; Adeniyi *et al.* 2018).Molecular characterization of mushrooms, particularly through DNA-based markers such as the Internal Transcribed Spacer (ITS) region of ribosomal DNA, has appeared as a reliable technique for species identification and phylogenetic analysis. This dual approach— incorporating morphological observations with molecular techniques— enhances the precision of species differentiation and helps in understanding the genetic variation within and between varieties and strains of *P. pulmonarius.*The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA) is widely distinguished as a universal DNA barcode marker for the identification of fungal species (Schoch *et al.* 2012 ; Kumar *et al.*2022).

This research work aims to perform both morphological and molecular characterization of *Pleurotus pulmonarius* to support its accurate identification, evaluate genetic variability, and contribute to strain selection and the development of improved cultivation practices for increasing yield and quality of mushroom.

MATERIALS AND METHODS

Sample Collection

Fruiting bodies of *Pleurotus pulmonarius* were collected from mushroom bags prepared in Mushroom research laboratory,Indira Gandhi Krishi Vishwavidyalaya (IGKV) Raipur, Chhattisgarh. Collected fruiting bodies were placed in sterile polypropylene bags and carried out to the research laboratory of Botany Department, Govt. Nagarjuna P.G. College of Science, situated on the Great Eastern Road, near Pt. Ravi Shankar Shukla University Campus,Amanaka, Raipur, capital of Chhattisgarh, for further analysis. Preliminary identification was based on macroscopic characteristics.

Morphological Characterization

Morphological characterization was performed by following the standards ofRajarathnam*et al.* **(**1987**),** andSenthilarasu and Kumaresan (2018).Fresh fruiting bodies were analyzed for morphological features such as pileus size, shape, color, margin, surface texture, gills spacing, stipe characteristics, stipe position and spore print color.

Cultural Characteristics

Healthy and mature fruiting bodies of *Pleurotus pulmonarius* were collected. The fruit bodies were surface sterilized using 70% ethanol, and inner tissue inner flesh was aseptically excised and inoculated onto Potato Dextrose Agar (PDA) plates supplemented with streptomycin (100 µg/ mL) to suppress bacterial contamination. The inoculated plates were incubated at 25/ ±/ 2/ °C for 7–10 days in a BOD incubator. Cultural characteristics such as mycelial growth,colony diameter, colony color, texture, margin type were recorded at regular intervals.

Culture and DNA Extraction

Tissue cultures were initiated by aseptically transferring inner flesh from fresh fruiting bodies of *P.pulmonarius* onto PDA plates. Plates were incubated at 25/ ±/ 2/ °C for 7–10 days. Mycelial mass was harvested from the growing edge and used for DNA extraction.Genomic DNA was extracted using the CTAB method with minor modifications. Mycelial tissue (~100 mg) was ground in liquid nitrogen and incubated in CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% polyvinylpyrrolidone) at 65°C for 30 minutes. The lysate was purified with chloroform: isoamyl alcohol (24:1) and centrifuged at 13,000 RPM for 3 minutes. DNA was precipitated using isopropanol and again centrifuged at 10,000 RPM for 3 minutes. Washed pellet with 70% ethanol and resuspended in TE buffer(Kumar *et al.* 2022).

PCR Amplification and Sequencing

The internal transcribed spacer (ITS)1-5.8S-ITS2 region of rDNA was amplified by using polymerase chain reaction (PCR) using universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC3') (White *et al.* 1990**)**. PCR reactions were carried out in a 25 µL volume containing 1× PCR buffer, 2.5 mM MgCl, , 0.2 mM dNTPs, 0.4 µM of each primer, 1 U Taq DNA polymerase, and 50 ng of genomic DNA of mycelium. The thermal cycling profile consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min.PCR products were electrophoresedon 1.5% agarose gel stained with ethidium bromide and visualized on a UV transilluminator. A negative Control containing no DNA template was included to check the presence of contamination in the reagents and reaction buffer. Positive amplicons were purified using a Qiagen PCR purification kit and sequenced bi-directionally using Sanger sequencing(Kumar *et al.*2022).

Sequence Analysis

Purified PCR products of ITS region were successfully amplified and subjected to Sanger sequencing using standard ITS primers on an ABI 3730xl DNA Analyzer. Both forward and reverse chromatograms were sequenced using Sequence Scanner Software v2.0. Trimming and alignment of forward and reverse reads were performed to generate a consensus sequence. The assembled ITS sequence was compared with existing sequences in the NCBI GenBank database using BLASTn for confirmation of species identity. The first ten sequences were selected Based on maximum identity score and aligned using the MEGA version 12. Phylogenetic analysis was performed using MEGA 12 with neighbor-joining (NJ) and maximum likelihood (ML) methods, with bootstrap analysis (1000 replicates) to appraise clade support.

RESULTS AND DISCUSSION

Morphological Characterization

The collected fruiting bodies of *Pleurotus pulmonarius* exhibited distinct typical macroscopic features of the species. The sporophore characters have been presented in Table 1.

Fruiting Body

Fruiting bodiesappearedmainly in clustered form,but single fruiting body also occurred on mushroom bags (Fig. 1).

Table1 : Sporophorecharacters of *Pleurotus pulmonarius*

S. No.	Pileus size (cm)	Stipe length (cm)	Stipe thickness (cm)
1.	6.2	3.3	3.3
2.	7.1	3.8	3.4
3.	7.9	3.9	3.8
4.	8.4	4.1	4.1
5.	11.3	4.6	4.3
Mean ± S.E.	8.18± 0.86	3.94±0.21	3.78±0.19

Table 2 : Growth and Cultural Characters of *Pleurotuspulmonarius*

Name of Mushroom	Radial growth (cm)			Nature of growth
	3 days	5 days	7 days	
Pleurotuspulmonarius	0.4	3.7	7.1	Absolute milky white colony, cottony texture, smooth and even margin,pigmentation absent, circular and uniform growth.

Table 3 : Sequence alignment of*Pleurotus pulmonarius*

Accession	Description
LT627807.1	*Pleurotus pulmonarius*
MN244436.2	*Pleurotus pulmonarius,* voucher JFRL28
MN244439.2	*Pleurotus pulmonarius,* voucher JFRL60
MN622710.1	*Calocera sinensis*, voucher HFJAU-TD082
OQ073787.1	*Pleurotus pulmonarius,* strain p1307

Pileus

Macroscopically, the pileus was convex to plane, ranging from 6–12 cm in diameter, with a smooth and shiny surface. At pin head stage pileus color was grey and when fruiting body was formed pileus color turned light grey brown or greyish white (Fig.2). The margin was incurved in young specimens, becoming wavy at maturity.

Stipe

The stipe was eccentric, cylindrical, fleshy fibrous, solid and white, measuring 3–5 cm in length and 3–5 cm in diameter (Fig.2).

Hymenium

In *Pleurotuspulmonarius*, hymenium was located on the gills (lamellae) underneath the mushroom cap. This layer contains specialized cells known as basidia, which produce basidiospores.

Lamellae

Lamella waswhite colored, turning cream colored with age,crowded and decurrent with closed gill spacing.

Spore Print

The spore print was white colored (Fig.3).The basidiospores of *Pleurotus pulmonarius* were

smooth, hyaline and thin-walled. They were cylindrical, ellipsoid, or oblong with rounded ends. The spores were produced on basidia, present over the gill surface and give a white spore print.

Cultural Characterization

The cultural characteristics of *Pleurotus pulmonarius* were studied after seven days of incubation at 25 ± 2°C on PDA medium. On PDA, the mycelium showed strand, circular growth, reaching the edge of the culture plate within 9-10 days. The colony was dense, cottony texture, even with a slightly irregular margin, raised with concentric rings,and absolute milky white colored(Table 2, Fig.4). Pigmentation was absent.

Molecular Characterization

The internal transcribed spacer (ITS) region of *Pleurotus pulmonarius* was successfully amplified and sequenced using standard ITS primers on an ABI 3730xl DNA Analyzer. Both forward and reverse chromatograms were obtained and analyzed using Sequence Scanner Software v2.0. The forward sequence (0623_023_001_PCR_6_ITS_F_H06.ab1) yielded 229 bases with a quality value (QV) e" 20, indicating high sequencing accuracy. Although the contiguous read length (CRL) was relatively short (47 bp), the average signal strength was 72, with balanced peak intensities for all four nucleotides (A: 62, T: 86, C: 75, G: 67).

The reverse chromatogram (0623_023_002_PCR_6_ITS_R_H06.ab1) yielded 627 bases with a quality value (QV) e" 20, indicating high sequencing accuracyand high-quality reads suitable for downstream analysis. The CRL was relatively long (621 bp), with an overall average signal strength was 266. The average peak intensities recorded for all four nucleotides were as follows: A: 219, T: 297, C: 257, G: 292. This balanced distribution of nucleotide peak intensities indicates a clean and high-quality signal detection chromatogram, reflecting that the sequencing data is suitable and reliable for accurate DNA analysis, including alignment and species identification. The PCR amplified product of ITS 5.8S rDNA of *Pleurotus pulmonarius* was approximately 630 base pairs in length. The amplified product was successfully sequenced, and the obtained consensus sequence was undergone to BLASTn analysis against the NCBI GenBank database. BLASTn analysis of the consensus sequence showed closest match with NCBI sequence number LT627807.1(100 %) in the NCBI GenBank database was found to be the *Pleurotus pulmonarius*. The query coverage was 100% with 99.84 percent identity with LT627807.1 (*P. pulmonarius*) and the e-value was 0.00, which showed that the sample was *Pleurotus pulmonarius*, thereby confirming morphological identification at the molecular level.

Phylogenetic analysis was conducted using the Neighbor-Joining method in MEGA 12. The resulting tree clustered the current isolate within the *Pleurotus pulmonarius* clade with strong bootstrap support. The molecular data supported the identification, classification and demonstrated the utility of ITS sequences in accurately identifying *Pleurotuspulmonarius* (Table 3, Fig.5).

Consensus Sequence of (630 bp)

CCTCTCCGTA GGTGAACCTG CGGAAGGATC
ATTAATGAAT TCACTATGGA GTTGTTGCTG

GCCTCTAGGG GCATGTGCAC GCTTCACTAG
TCTTTCAACC ACCTGTGAAC TTTTGATAGA

TCTGTGAAGT CGTCCTTCAA GTCGTCAGAC
TTGGTTTGCT GGGATTTAAA CGTCTCGGTG

TGACAACGCA GTCTATTTAC TTAACACACC CCAAATGTAT GTCTACGAAT GTCATTTAAT GGGCCTTGTG CCTATAAACC ATAATACAAC TTTCAACAAC GGATCTCTTG GCTCTCGCAT

CGATGAAGAA CGCAGCGAAA TGCGATAAGT AATGTGAATT GCAGAATTCA GTGAATCATC

GAATCTTTGA ACGCACCTTG CGCCCCTTGG TATTCCGAGG GGCATGCCTG TTTGAGTGTC

ATTAAATTCT CAAACTCACA TTTATTTGTG ATGTTTGGAT TGTTGGGGGT TGCTGGCTGT

AACAAGTCGG CTCCTCTTAA ATGCATTAGC AGGACTTCTC ATTGCCTCTG CGCATGATGT

GATAATTATC ACTCATCAAT AGCACGCATG AATAGAGTCC AGCTCTCTAA TCGTCCGCAA

GGACAATTTG ACAATTGACC TCAAATCAGG

The integration of morphological, cultural and molecular characteristics provided a strong approach for precise identification of *P. pulmonarius*. Morphological traits are useful for initial identification and classification, they can be also influenced by environmental factors and substrate conditions, often leading to misidentification, especially among closely related species of *Pleurotus*. Molecular characterization using ITS sequencing served as a reliable tool for confirming species identity and provide sufficient resolution for resolving taxonomic uncertainties.

Morphological characteristics observed as pileus colour, shape, gill arrangement, stipe position, and basidiospore features are important taxonomic parameters for mushroom identification. Similarly, cultural characteristics like colony colour, mycelial texture, density and radial growth pattern also important parameter. These findings indicate the existence of species-specific variability and are in agreement with Pagoti*et al.* (2018) reporting that morphological and cultural characteristics are useful for preliminary characterization of *P. pulmonarius*.

In addition to morphological and cultural observations, molecular characterization provided more reliable confirmation of species identity and genetic variability. Molecular analysis supported the conventional characterization methods and helped in differentiating closely related *Pleurotus* species. Since environmental factors may

Fig 2: Fruiting Body of *Pleurotuspulmonarius*(a) Pileus(b) Gills

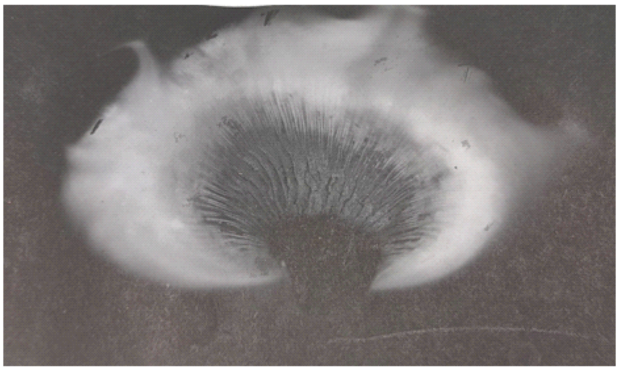

Fig.3 : Spore print of *Pleurotuspulmonarius*

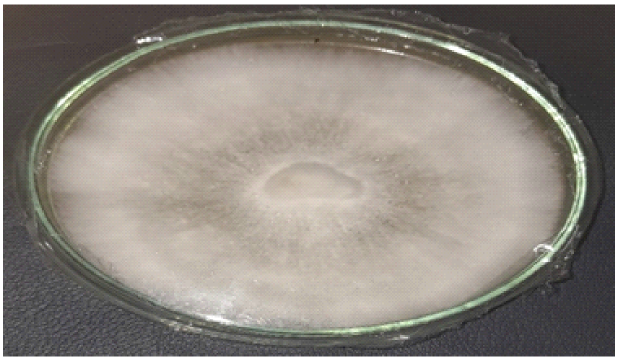

Fig 4 : Pure cultureof *Pleurotuspulmonarius*

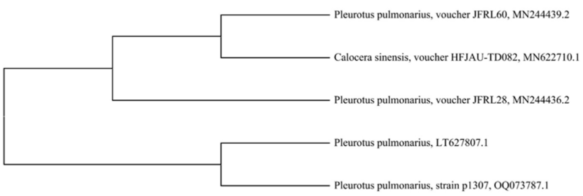

Fig 5: Genetic relatedness of *Pleurotuspulmonarius*

influence morphological expression, molecular techniques serve as effective tools for accurate species authentication, maintenance of genetic purity, and strain identification. The generated molecular data also provide valuable baseline information for future studies related to taxonomy, phylogenetic relationships, and strain improvement programs. Bernardo *et al.* (2004) identified *P. albidus, P. cystidiosus, P. ostreatus, P. pulmonarius, P. rickii* and *P. djamor* on the basis of macro- and micromorphological characteristics in Argentina. Kumar *et al,* (2022) worked on morphological and molecular characteristics of *Agaricus bisporus, Calocybe indica*, *Flammulinavelutipes*, *Pleurotus florida* and *Volvariella volvacea*. Chaudhary and John (2017) studied the morphological and molecular characteristics of *Pleurotus cystidiosus*.

Overall, the integration of morphological, cultural, and molecular characterization provided a comprehensive understanding of the studied *Pleurotuspulmonarius*. The present study also established important baseline data for *Pleurotus pulmonarius* under the agro-climatic conditions of Chhattisgarh. The findings of this investigation may be useful for future research, sustainable mushroom cultivation, development of superior strains, and effective utilization of agricultural wastes for nutritional and economic benefits.

CONCLUSION

The present research successfully identified and confirmed *Pleurotus pulmonarius* through an integrated technique using both morphological and molecular characterization. The macroscopic traits were compatible with the known characteristics of the *Pleurotus pulmonarius*. Molecular analysis using ITS region sequencing further confirmed the morphological identification, with high similarity to reference sequences MN244439.2 in the GenBank database and strong clustering within the *P. pulmonarius* clade in phylogenetic analysis.

This study established complete baseline information on the morphological, cultural and molecular characteristics of *Pleurotus pulmonarius* under the agro-climatic conditions of Chhattisgarh. Detailed evaluation of fruiting body morphology, cultural characteristics, and molecular aspects provided a systematic characterization of the *Pleurotus pulmonarius* and generated valuable report for future scientific research.

Although the sporophores used in the investigation were procured from controlled cultivation conditions instead of wild habitats, the characterization conducted in this study remains scientifically significant. Controlled environmental conditions for growth minimize external variability and enable accurate observation of stable species-specific characteristics. Therefore, the collected information is important for establishing authenticated reference dataof*Pleurotus* *pulmonarius*, which can be used in future studies related to strain authentication, maintenance of genetic purity, molecular taxonomy and phylogenetic analysis.

Furthermore, the molecular characterization performed in this research provides a strong foundation for breeding and future strain improvement. The collected baseline molecular data may support the identification of superior cultivated varieties possessing desirable features such as rapid growth, enhanced yield, environmental adaptability and improved nutritional quality. In addition, this work provides valuable information for germplasm conservation and biotechnological applications of *Pleurotus pulmonarius*.

Overall, the present study not only enhances the scientific understanding regarding*Pleurotus pulmonarius* but also provides a useful base for future research, commercial cultivation and sustainable mushroom production in Chhattisgarh and similar agro-climatic regions.

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DECLARATION

Conflict of interest. Authors declare no conflict of interest.

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