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Morphological and Molecular Characterization of Oyster Mushrooms of Kerala

Krishnapriya, P. J.[1*], Geetha, D.[2] and Priya, R. U.[3]

[1,3]PhD. Scholar, [2]Professor,

Department of Plant Pathology, College of Agriculture, Vellayani, Kerala, India

*Corresponding Author E-mail: krishnasaketham@gmail.com

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ABSTRACT

Pleurotus mushroom, generally referred as the 'oyster mushroom'is the well appreciated one in Kerala, for its broad adaptability under diverse agro-climatic conditions. There are 15 to 20 species of oyster mushrooms, available from Kerala, of which the common ones are Pleurotus eous and Pleurotus florida. P. eous has soft, pink coloured sporophores. P. florida has decurrent, pure white and fleshy sporophores. New additions to the Pleurotus family, are Pleurotus opuntiae and Pleurotus cystidiosus. Sporocarps of P. opuntiae are stark white, delicately fringed at the edges, borne on short stalks. P. cystidiosus has bigger size, thick, fleshy, buff coloured and less spore forming sporocarps. The oyster mushrooms were isolated from the natural locations of Kerala and brought in to pure cultures. P. eous, P. florida, P. opuntiae and P. cystidiosus took 9.0, 7.3, 7.0 and 24.7 days for completing the growth in petriplates, when potato dextrose peptone agar was used for culturing. The culture of P. cystidiosus is characteristic, due to the occurrence of black coremial structures, indicating its anamorph stage (Antromycopsis broussonetiae). The morphological identities of the species were confirmed by ITS sequencing and registered at Genbank database (accession numbers: KY214257, KY214256, KY214255, KY214254). A phylogenetic tree was also constructed using Clustal W software, which grouped the four oyster species to three clusters. Here, P. florida and P. eous was found in the same cluster, indicating its common evolutionary lineage.

Keywords: *Oyster mushrooms, Morphological, Cultural, ITS sequencing, Phylogenetic analysis*

INTRODUCTION

Mushroom is a macrofungus with a distinctive fruiting body which can be either epigeous or hypogeous and large enough to be seen with naked eye and picked by hand[3]. Mushrooms have long been appreciated as an important source of bioactive compounds of medicinal value. The nutritional value of mushrooms depends on their chemical composition. *Pleurotus* mushroom, generally referred as the 'oyster mushroom'or 'Dhingri', is the well appreciated one in Kerala, for its broad adaptability under diverse agro-climatic conditions.

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Oyster mushrooms are generally considered nutraceutical and are rich in anti-oxidants, dietary proteins, fibres, vitamins and minerals. There are 15 to 20 species of oyster mushrooms available for cultivation in Kerala, of which the popular ones are *Pleurotus eous and Pleurotus florida.* New additions to the *Pleurotus* family, are the newly identified *Pleurotus opuntiae and P. cystidiosus.*

Different species of oyster mushrooms are still distinguished, based on the colour, texture and taste of fruiting bodies. Only few species are identified at genus and species level. The use of molecular tools, is almost essential to ensure that the inoculum used is from the correct species. Molecular tools provide more accurate methods for identification, than the few characters afforded by the traditional morphological features[2].

Molecular markers, PCR (Polymerase Chain Reaction) and non-PCR based, are the widely used techniques for mushroom identification and characterization. However, direct sequencing of PCR product of repetitive nuclear DNA of mushrooms is a powerful tool for identification and phylogenetic studies[5]. ITS (Internal Transcribed Spacer) refers to a piece of non-functional RNA situated between structural ribosomal RNAs on a common precursor transcript. Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because, it is easy to amplify small quantities of DNA and has a high degree of variation between closely related species. Thus ITS region is the most widely used marker to sequence the DNA region in fungi[11]. The present study describes the nucleotide sequencing of four cultivated oyster mushrooms collected from Kerala, India, using genomic DNA from fruit bodies. The rDNA-ITS (Ribosomal DNA Internal Transcribed Spacers) fragments of the genomic DNA were amplified using ITS specific universal primers. The nucleotide sequences of the four mushrooms were matched from the available known sequences of GenBank database. Also, a phylogenetic tree was constructed using Clustal W software (Neighbor Joining method), showing interrelationship among the oyster species.

MATERIALS AND METHODS

Isolation, morphological and cultural characterisation of mushrooms

Mushrooms were isolated by adopting the tissue culture method. Medium sized healthy mushroom sporocarp of each mushroom species were collected from their natural locations. The mushroom sporocarp was surface sterilized by wiping it with cotton dipped in 70% ethyl alcohol. The mushroom was split open longitudinally and a small bit of the tissue from the exposed surface was scooped out with the help of a sterile forceps and transferred to Potato Dextrose Agar (PDA) slants under aseptic conditions and incubated under room temperature (28±4°C) for 4 days. The growth obtained from this was then purified by the hyphal tip method and maintained on PDA slants by subculturing. The morphological and cultural characters were examined and classified according to taxonomic keys and descriptions[1].

Molecular characterisation of mushrooms

DNA isolation using NucleoSpin® Plant II Kit (Macherey-Nagel)

Mycelium (100mg) is homogenized using liquid nitrogen and the powdered tissue is transferred to a microcentrifuge tube. Rest of the steps in DNA isolation, was done as per the manufacturer's guidelines. The eluted DNA was stored at 4^{o}C.

Agarose Gel Electrophoresis for DNA Quality check

The quality of DNA isolated was checked, using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until, bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume, which contained 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers.

Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
ITS	ITS-1F	Forward	TCCGTAGGTGAACCTGCGG
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile

98 °C	-	30 sec	
98 °C	-	5 sec	40 cycles
60 °C	-	10 sec	
72 °C	-	15 sec	
72 °C	-	60 sec	
4 °C	-	∞	

Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded. Electrophoresis was performed at 75V power supply, with 0.5X TBE as electrophoresis buffer, for 1-2 hours, until bromophenol blue front has migrated, to the bottom of gel. The molecular standard used, was 2-log DNA ladder (NEB). Gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) (Figure 2).

ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consisted of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer, for the removal of unwanted primers and dNTPs from a PCR product mixture. 5 µl of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes, followed by enzyme inactivation at 80°C for 15 minutes.

Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA), following manufactures protocol. The PCR mix consisted of the following components: PCR Product (ExoSAP treated)-10 to 20 ng, primers- 3.2 pM (either Forward or Reverse), sequencing mix-0.28 µl, 5x Reaction buffer-1.86, sterile distilled water- made up to 10µl. Sequencing PCR temperature profile, consisted of a 1st cycle at 96°C for 2 minutes, followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes for all the primers.

Post Sequencing PCR Clean up

Master mix I of 10µl milli Q and 2 µl 125mM EDTA is made per reaction. 12µl of master mix I is added to each reaction containing 10µl of reaction contents and are properly mixed. Master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol per reaction is made. 52 µl of master mix II is added to each reaction. Contents are mixed by inverting and incubated at room temperature for 30 minutes. Contents are spinned at 14,000 rpm for 30 minutes. Supernatant is decanted, 100 µl of 70% ethanol is added and spinned at 14,000 rpm for 20 minutes. Supernatant is decanted and repeat the 70% ethanol wash. Supernatant is again decanted and pellet is air dried. The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1.

Characterisation

The morphological characterisation of oyster mushrooms, was confirmed by performing a similarity search using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) database and the sequences (Figure) were matched with existing NCBI database. Multiple nucleotide sequence alignment and phylogenetic analysis was done using Clustal-X software. Sequence phyllograms were constructed using Phylip package and unrooted trees were generated using TreeView software.

RESULTS

Isolation of mushrooms

P. eous, *P. florida*, *P. opuntiae*, and *P. cystidiosus* were isolated from coconut, jack, arecanut and asoka (*Saraca asoca* (Roxb.)) tree logs respectively, from Vellayani, Kerala, India. Isolates were subsequently brought in to pure cultures.

Morphological and cultural characterisation of mushroom species

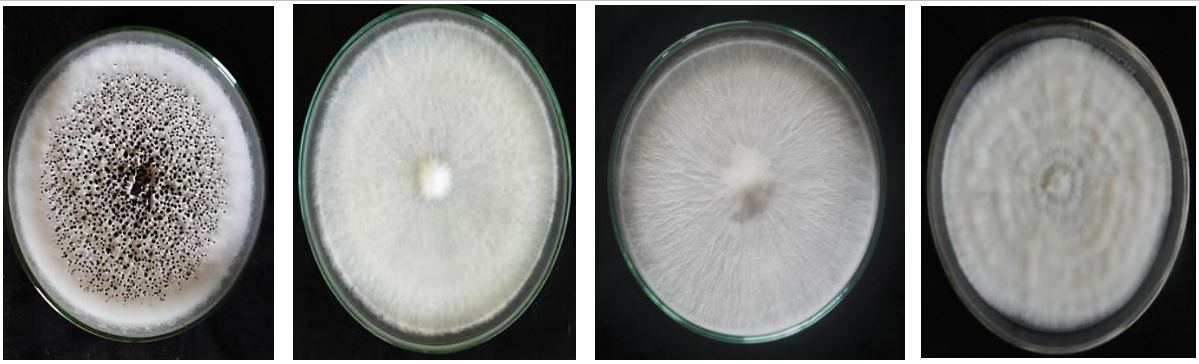
P. eous has pink coloured sporophores, soft when young and medium hard at maturity. *P. florida* has decurrent, pure white and fleshy sporophores. Sporocarp masses of *Pleurotus opuntiae* looked so much like angelic wings flapped around the mushroom beds. Sporocarps are stark white, delicately fringed at the edges, borne on short stalks. Also, a characteristic thick white patch can be seen at the centre of sporocarps. *Pleurotus cystidiosus* O.K.Mill (abalonus/brown oyster mushroom), is unique among the oyster mushrooms, owing to its bigger size, thick, fleshy, buff coloured, less spore forming sporocarps with the longest shelf life and lifecycle among the oyster species (Figure 1).

Pleurotus eous took 9.00 days for completing its growth on potato dextrose peptone agar (PDPA) in petriplate and produced pinkish white, thick mycelial growth. *Pleurotus florida* completed its growth in 7.30 days on PDPA and produced pure white, thick mycelial growth. *Pleurotus opuntiae* took 7.00 days for completing growth on PDPA and produced pure white, stranded mycelial growth. The thick white mycelium of *P. cystidiosus* was characteristic, due to the occurrence of black coremial structures, indicating its anamorph stage (*Antromycopsis broussonetiae*) (Table 1; Figure 2).

Similarly, many mushrooms were successfully identified based on their morphological and cultural characters by several other workers[4,6].

Table 1: Average growth time and culture characters of mushroom species

Sl.no	Name of Mushroom	Time for completion of growth (days)	Nature of growth
1.	*Pleurotus eous*	9.0	Pinkish white, thick mycelium
2.	*Pleurotus florida*	7.3	Pure white, thick mycelium
3.	*Pleurotus opuntiae*	7.0	White, stranded mycelium
4.	*Pleurotus cystidiosus*	24.7	Pure white, thick mycelium with numerous black, coremia



P. cystidiosus ***P. florida*** ***P. opuntiae*** ***P. eous***

Fig. 1: Cultural characters of *Pleurotus* species

P. opuntiae

P. cystidiosus

P. florida

P. eous

Fig. 2: Morphological characters of *Pleurotus* species

ITS sequenzing characterisation of oyster mushrooms

PCR products from the ITS specific primers, provided a clear single band around 600 bp (Figure 3 &4). The PCR product obtained was sequenced and sequence was analysed using BLAST software. Morphological identity of *P. cystidiosus* was confirmed as *P. cystidiosus subsp. abalonus* and registered in Genbank database (accession no: KY214254). Similarly, the other oyster species were identified as *P. eous*, *P. florida*, *P. opuntiae* and registered in Genbank database with accession numbers: KY214257, KY214256, KY214255, respectively (Figure 5). The determined nucleotide sequences of the mushrooms can provide additional information, enriching GenBank database, enabling molecular taxonomy and facilitating its domestication for humankind. In the present study, both morphological and molecular data were corroborating to each other. Lee *et al*.[8] identified three medicinal mushroom (*Ganoderma lucidum*, *Coriolus versicolor*, and *Fomes fomentarius*) from Korea, based on the nuclear large subunit rDNA sequences. Nucleotide sequencing of *Termitomyces albuminosus*[10], *Ganoderma lucidum; Agaricus bisporus*[9] were also performed, using ITS specific primers.

Morphological traits do not provide a meaningful frame work for evolutionary classifications. In this context, phylogenetic tree was done, which grouped the four oyster species to three clusters, signifying distinct evolutionary lineages (Figure 6). Here, *P. florida* and *P. eous* was found in the same cluster, indicating its common evolutionary lineage. Similarly, Khan *et al*.[7] have demonstrated that, ITS analysis and morphological evaluation are useful for characterization, genetic diversity analysis and identifying relationships among *Pleurotus* species of mushrooms.

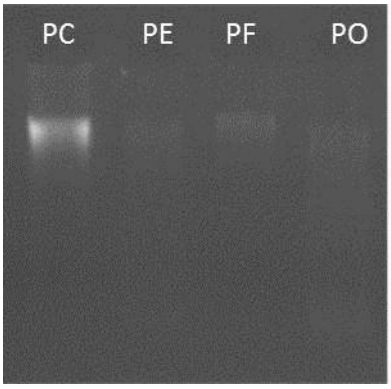


Fig. 3: DNA from *Pleurotus* species

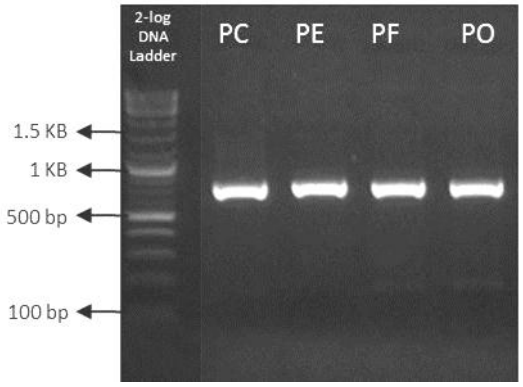


Fig. 4: PCR amplicons from *Pleurotus* species

>KY214254

AAGGATCATTAATGAATTCACTCATGAAGCTGATGCTGGTCTCTCGGGACATGTGCACGCTTCATA
AGTACATTCAACCACTTGTGCACTTTTGATAGATTCGCAGAGTTGCCCTCTCAGGTCAGTAAATGA
CTTGGTTGGTCGGGATTGTCACAGTCCTGGCTTTGACTTTGTGGGTCTATTATCTTATACACACTTG
TATGTCCATGAATGTTATTTTCTTGGGCCATGTGCCTATAAAACCTAATACAACTTTCAACAACGG
ATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT
CAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTTG
AGTGTCATTAAATTCTCAAATCTATAGAGCTTTTTTGTGATATAGATTTGGATTGTTGGGGGCTGCT
GGCTTTTTACCAAGTTGGCTCCTCTTAAATGCATTAGCGGGACTTTATTGCCTCTGCGCACAGTGTG
ATAATTATCTACGCTGGCCGACATGCAATGACTTTACAAGTCCAGCTTTCTAACTGTCTTTCAAGAC
AATGACTTGACAATTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAA

> KY214257

AAGGATCATTAATGAATTCACTATGGAGTTGTTGCTGGCCTCTAGGGGCATGTGCACGCTTCACTA
GTCTTTCAACCACCTGTGAACTTTTGATAGATCTGTGAAGTCGTCTCTCAAGTCGTCAGACTTGGTT
GCTGGGATTTAAACGTCTCGGTGTGACTACGCAGTCTATTTACTTACACACCCCAAATGTATGTCT
ACGAATGTCATTTAATGGGCCTTGTGCCTTTAAACCATAATACAACTTTCAACAACGGATCTCTTG
GCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTTGAGTGTCAT
TAAATTCTCAAACTCACTTTGGTTTCTTTCCAATTGTGATGTTTGGATTGTTGGGGGCTGCTGGCCT
TGACAGGTCGGCTCCTCTTAAATGCATTAGCAGGACTTCTCATTGCCTCTGCGCATGATGTGATAA
TTATCACTCATCAATAGCACGCATGAATAGAGTCCAGCTCTCTAATCGTCCGCAAGGACAATTTGA
CAATTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAA

> KY214256

TAATGAATTACAAAAGCTTTTGAAGTTGTTTTGCTGGTCTCTAGGGACATTGTGCACGCTTCATTAG
TTTCCACTTCATACCCCTGTGCACCTTTGATAGATTTCGGTTTGGGTTATCCTTTGGTTTTTTTTCCT
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ATAATGAATGGTTTATAATGACAAGGCCATGAGCCTTATAAACTTAATACAACTTTCAACAACGGA
TCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
AGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGA
GTGTCATTAAATTCTCAAACTCTATGACTTTATTGTTGTAGCTGTTTGGATTGTTGGGGGTTGCTGG
CTTCTTTCTTTGAAGTCGGCTCCTCTTAAATGCATTAGCGGGACTTTGTTGCCTCTGCGCATAGTGT
GATAATTATCTACGCTAGACGCATGCAATTCTTATAGTTGTCCAGCTTTCTAATCGTCTCAAGGGAC
AATTACTTTGACAATTTGACCTCAAA

> KY214255

AAGGATCATTAATGAATTACAAAAGCTTTTGAAGTTGTTTTGCTGGTCTCTAGGGACATTGTGCAC
GCTTCATTAGTTTCCACTTCATACCCCTGTGCACCTTTGATAGATTTCGGTTTGGGTTATCCTTTGGT
TTTTTTTCTTAATTGAAAGGCCTTTGGTTTCCTTAAAACGACTTCTATACTATACCACACACCAAAT
GTATGTTTTATAATGAATGGTTTATAATGACAAGGCCATGAGCCTTATAAACTTAATACAACTTTC
AACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCGAAGGGCAT
GCCTGTTTGAGTGTCATTAAATTCTCAAACTCTATGACTTTATTGTTGTAGCTGTTTGGATTGTTGG
GGGTTGCTGGCTTCTTTCTTTGAAGTCGGCTCCTCTTAAATGCATTAGCGGGACTTTGTTGCCTCTG
CGCATAGTGTGATAATTATCTACGCTAGACGCATGCAATTCTTATATTGTCCAGCTTTCTAATCGTC
TCAAGGGACAATTACTTTGACAATTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAA

Fig. 5: ITS sequences of *Pleurotus* species

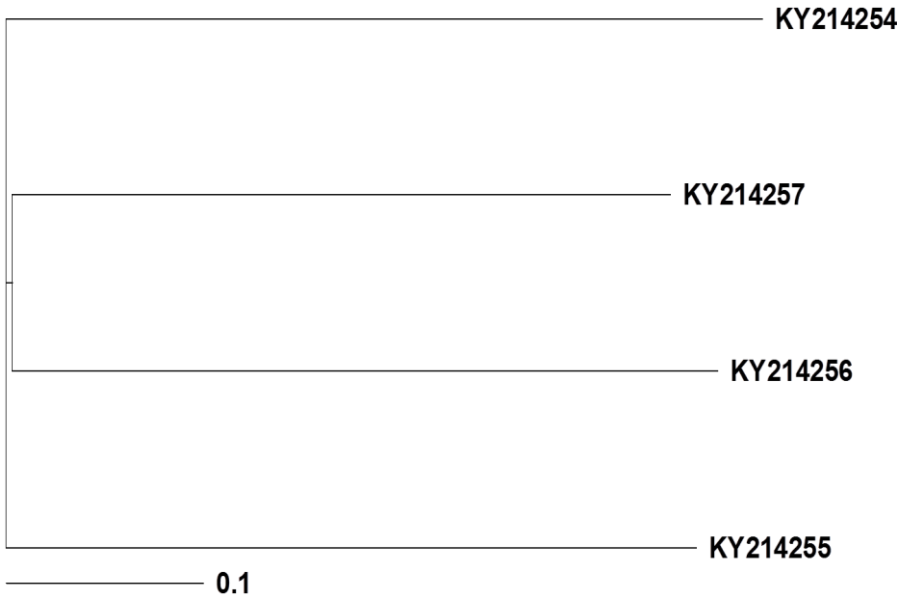

Fig. 6: Dendrogram based on ITS sequences of *Pleurotus* species

CONCLUSION

In this study, a preliminary work on the morphological, cultural and molecular characterization of oyster mushrooms from Kerala, was done. Studies identified the mushrooms as *P. eous*, *P. florida*, *P. opuntiae* and *P. cystidiosus* subsp. *abalonus*. The determined nucleotide sequences based on ITS sequencing and further phylogenetic study, provided information, enriching the GenBank database. The present work can pave the way, for further molecular and evolutionary comparison among species cultivated/available worldwide, enabling efficient cultivation of mushrooms for human benefits.

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