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Original Research

Morphotaxonomic and Phylogenetic Study of *Pleurotus cystidiosus* isolated from *Madhuca longifolia* from Western Ghats, India

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Abstract

This study documents the occurrence of *Pleurotus cystidiosus*, a pleurotoid basidiomycete repeatedly recorded and isolated from crevices on the living trunk of *Madhuca longifolia* over three consecutive monsoon seasons, consistently emerging from the same substratum of the host tree. The species' identity was confirmed using a polyphasic approach that integrated detailed morphological observations, cultural characteristics, and multilocus phylogenetic analyses based on ITS and LSU rDNA sequences. This integrative approach provides a reliable framework for accurate taxonomic identification, particularly for morphologically complex agaricomycetes. Re-documentation of an important taxon that includes sexual and asexual morphs serves as a useful reference for the next generation of researchers, demonstrating how classical morphology can effectively complement molecular tools.

Keywords: Fungi, Pleurotaceae, Phylogeny, Epigeous, Taxonomy, India.

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1. Introduction

The genus *Pleurotus* (oyster mushroom) encompasses approximately 521 species, as recorded in Index Fungorum (accessed 1st January 2026). Among these, edible taxa such as *P. ostreatus* and *P. eryngii* dominate global cultivation due to their culinary appeal, nutritional profile, and medicinal potential. These mushrooms feature distinctive fan- or oyster-shaped caps and a pleasant texture, rendering them staples in diverse cuisines. *Pleurotus ostreatus* first gained prominence during World War I in Germany as a famine food, and has since evolved into one of the world's key edible fungi owing to its palatability, nutrient density, and ease of cultivation (El-Batal *et al*., 2015).

Nutritionally, *Pleurotus* spp. offer high protein levels (up to ~20% in *P. ostreatus*, rivalling conventional sources), essential amino acids, vitamins, minerals, dietary Fiber, and antioxidants, supporting their role in human diets (Li *et al*., 2017; Oyetayo & Ariyo, 2013; Jayakumar *et al*., 2011). *P. eryngii* (king oyster mushroom) stands out for its firm texture and umami flavor, driving demand in gourmet and functional foods (Ma *et al*., 2020; Gao *et al*., 2022). Economically, *Pleurotus* ranks as the second most cultivated mushroom genus, accounting for ~25% of global production (Selvaananthi & Alphonse, 2022; Bijla & Sharma, 2023; Ragini *et al*., 2025). Its cultivation is cost-effective and versatile, thriving on lignocellulosic wastes such as straw and sawdust, thereby promoting sustainable waste valorization and aligning with circular economy principles (Zied *et al.,* 2020; Ogbu *et al*., 2021; Silva *et al*., 2024).

Beyond nutrition, *Pleurotus* spp. holds biotechnological promise. *P. ostreatus* produces ligninolytic enzymes (e.g., laccases) for degrading aromatic compounds, with applications in pulp/paper, textiles, and bioremediation (El-Batal *et al.,* 2015; Chamutpong *et al.,* 2019). Medicinal attributes—including immunomodulation, antioxidation, anticancer potential, hypoglycemia, cardioprotection, and hypocholesterolemia—position them for nutraceutical and pharmaceutical uses (Joh *et al*., 2007; Jayakumar *et al*., 2011; Rout *et al.,* 2005).

Contemporary taxonomy employs polyphasic approaches that integrate morphology with molecular phylogenetics. Ribosomal DNA regions, notably ITS and LSU, resolve cryptic diversity and species complexes, such as *P. ostreatus*, *P. pulmonarius*, and *P. cystidiosus*, via multilocus analyses and genealogical concordance phylogenetic species recognition (Bao *et al.,* 2004; Zervakis *et al.,* 2004; Li *et al*., 2020). Sampling from understudied regions (e.g., Southeast Asia, Africa, and the Indian subcontinent) has revealed novel species and strains, informing breeding, conservation, and assessments of genetic/chemical diversity for nutritional and therapeutic applications (My, 2018; Lin *et al*., 2022).

Combined ITS-LSU markers delineate clades and ecological adaptations, enhancing identification and underscoring the ecological/economic roles of *Pleurotus* (Wang *et al*., 2008; Zervakis *et al*., 2013; Naguib *et al*., 2014). Despite advances, *Pleurotus* taxonomy remains dynamic amid new lineages and phylogenomic needs. The present study documents the sexual as well as asexual morphs of *P. cystidiosus* found during this study.

2. Materials and Methods

2.1. ***Collection and morphological identification of basidiocarps***

Fresh basidiocarps emerging from crevices of a living *M. longifolia* trunk were collected in paper bags from the premises of the Agharkar Research Institute, Pune, Maharashtra, India (Fig. 1),

and transported to the laboratory. Field photographs of fresh specimens were taken, and notes were recorded on habit, habitat, colour, odour, and colour changes using the colour codes of Kornerup and Wanscher (1978). Macroscopic characters were documented from fresh basidiomata. Schäffer's reaction and KOH reaction were tested on the surface of fresh basidiomata. Monosporic culture was raised by spreading spores onto potato dextrose agar plates. Colony characteristics were recorded after 14 days of incubation at 25 °C.

For microscopic examination of basidiocarps, sections were prepared from freshly collected samples and mounted in lactophenol cotton blue. Diagnostic features of basidiospores, basidia, cystidia, and pileipellis, etc., were observed using lactophenol cotton blue as both staining cum mounting medium under an Olympus BX53 microscope (Tokyo, Japan). Measurements and photomicrographs of fungal structures were recorded using Olympus cellSens Standard 3.2 software and an Olympus DP74 camera attached to the microscope. Additional microphotographs were taken using a scanning electron microscope (Zeiss MA15, Jena, Germany). Basidiospore measurements include the full range of spore dimensions, the range of the spore quotient (Q=length/width), and its mean value (Qm). The specimen (dried Herbarium specimen) has been deposited at the Ajrekar Mycological Herbarium (AMH 10819), and the living culture has been deposited at the National Fungal Culture Collection of India (NFCCI 6265).

2.2. DNA extraction and Sequencing

Genomic DNA was isolated from freshly collected *Pleurotus* basidiomes using a modified method of Aamir *et al.* (2015). Approximately 300 mg of tissue was pulverized in liquid nitrogen using a mortar and pestle. Subsequently, 1 mL of lysis buffer (100 mM Tris-HCl (pH 8), 50 mM EDTA (pH 8), and 3% SDS) was added, and the mixture was incubated at 65°C for 5 minutes. The mixture was later transferred to a fresh Eppendorf tube and centrifuged at 13,000 rpm for 15 minutes at room temperature. The resulting supernatant was carefully transferred to a fresh microcentrifuge tube, and an equal volume of phenol:chloroform:isoamyl alcohol was added. The mixture was shaken well, then centrifuged at 13,000 rpm for 10 minutes.

The upper aqueous layer was transferred into a fresh microcentrifuge tube, and an equal volume of chilled isoamyl alcohol (stored at -20°C) was added. This mixture was incubated for 20 minutes. The DNA precipitated, and the microcentrifuge tube was centrifuged at 13,000 rpm for 10 minutes to pellet down the DNA. The supernatant was discarded, and the DNA pellet was washed with 70% ethanol, then centrifuged at 13,000 rpm for 5 minutes. The pellet was allowed to air-dry and later dissolved in 1× TE buffer [10 mM Tris-HCl, 1 mM EDTA]. To eliminate RNA, 1 µL of RNase A Solution (20 mg/mL) was added, followed by vortexing and incubation at 37°C for 30 minutes. The quality and integrity of the extracted genomic DNA were assessed using 0.8% agarose gel electrophoresis (Sigma-Aldrich, USA) prepared in 1× TAE buffer (0.4 M Tris acetate, 0.01 M EDTA) containing 0.5 µg/mL ethidium bromide.

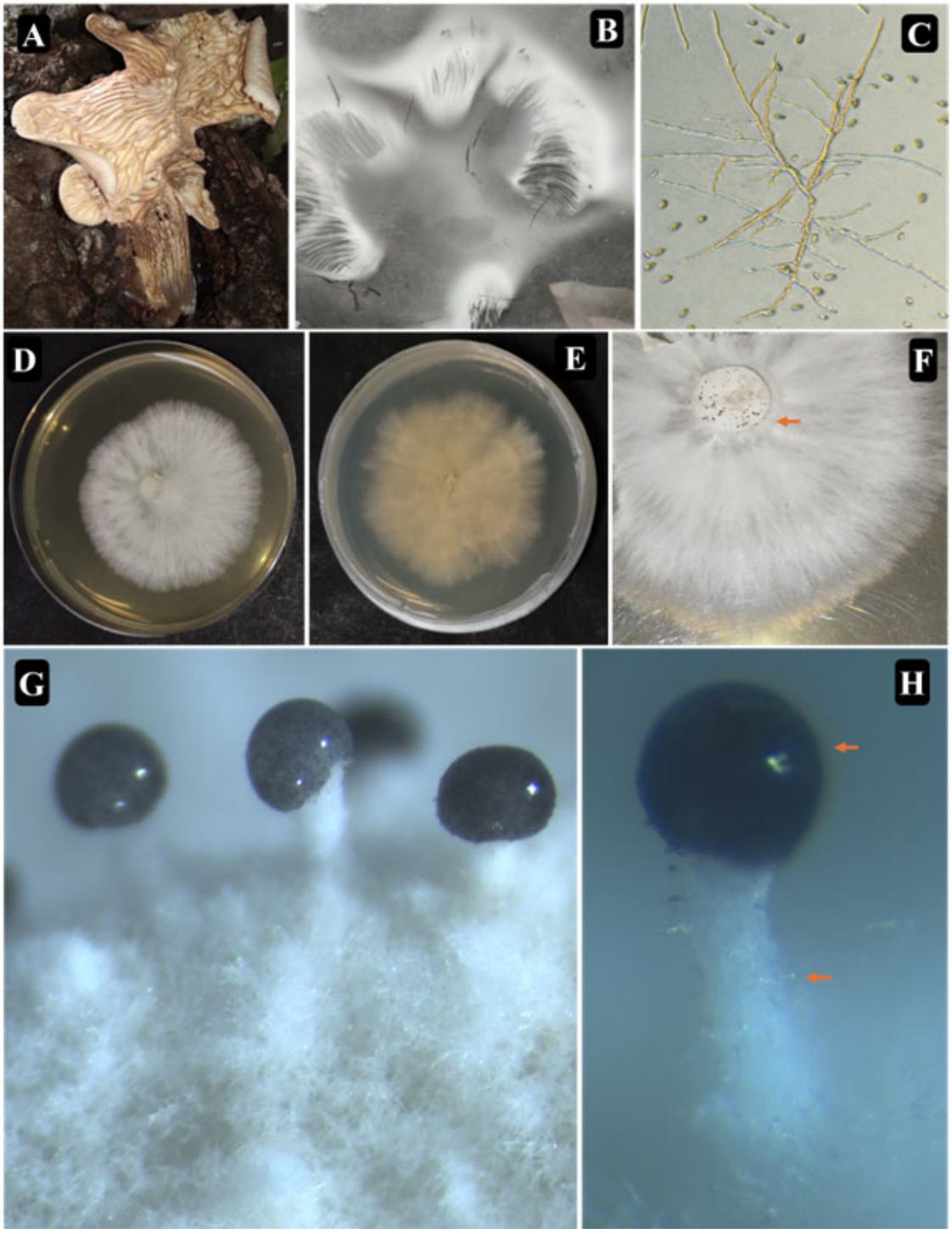

Figure 1. *Pleurotus cystidiosus* (AMH 10819). A. Photograph of sporophore with host, showing upper surface and the under-surface showing attachment of lamellae. B. Spore print, C. Germinated basidiospores, D–E. Reverse and Front view of colony on PDA medium, F. Growing colonies (centre of colony arrow showing blackish pin head like coremia (showing arrow), G–H Numerous developing coremia of asexual morph (*Antromycopsis macrocarpa*) with floccose colonies (arrow showing glazed black head with white myceloid stalk).

Partial gene sequences of isolates were determined for two gene markers ITS and LSU rDNA. The primer sets used to amplify particular gene regions are ITS-5 (5'-GGAAGTAAAAGTCG-TAACAAGG-3') and ITS-4 (5'- TCCTCCGCTTATTGATATGC-3') for amplifying and sequencing Internal transcribed spacer (ITS) (White *et al*., 1990); LR-OR (5'-ACCCGCTGAACTTAAGC-3') and LR-7 (5'-TACTACCACCAAGATCT-3') for amplifying and sequencing 28S large subunit of the nrDNA (LSU) (Vilgalys *et al*., 1990; Vilgalys *et al*., 1994).

PCR was performed in a 25 µL reaction using the thermocycling conditions described earlier by Rana and Singh (2025). The PCR amplicons were purified using a PCR purification kit (Genei Laboratories Pvt Ltd, Bangalore, India) according to the manufacturer's instructions. Purified PCR products of all marker genes were checked on a 1.2% agarose gel electrophoresis stained with 0.5 µg/mL ethidium bromide. They were further subjected to a sequencing PCR reaction using a BigDye®Terminator v3.1 Cycle Sequencing Kit, as per the manufacturer's instructions, as described earlier by Rana & Singh (2025). Sequences obtained were submitted to NCBI GenBank.

2.3. Phylogenetic analysis

To determine the evolutionary relationship of this taxon, two gene regions, ITS and LSU, were used to compare with the existing species under the genus *Pleurotus*. The sequences of the related authentic strains were retrieved from NCBI. A phylogenetic tree was constructed using 28 *Pleurotus* sequences, aligned with sequences of this taxon. *Hohenbuehelia mastrucata* TRTC 152314 and *Hohenbuehelia valesiaca* Roux 2975 were selected as the outgroup taxa. The strains used for the construction of the phylogenetic tree, along with their accession numbers and other details, are tabulated in Table 1. Each gene region was aligned individually with MAFFT v. 6.864b (Katoh & Standley, 2013). The alignments were adjusted and manually checked with Aliview (Larsson, 2014). Further, alignments were concatenated using MEGA11 (Tamura *et al*., 2021) and used for the phylogenetic analyses. The best substitution model was figured using ModelFinder (Kalyaanamoorthy *et al*., 2017). Later, the Windows version IQ-tree tool v.1.6.12 (Nguyen *et al*., 2015) was used to construct the phylogenetic tree. Bootstrap analyses of 1000 replicates assessed the reliability of the branches. The constructed phylogenetic tree was visualized in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/ accessed on 18th August 2025)

Table 1. GenBank accession numbers and other details of the isolates used in the phylogenetic study.

No	Species	ID (status, strain, isolate, voucher)	Country, isolation source	ITS	LSU
1	*Hohenbuehelia mastrucata*	TRTC 152314^{T}	-	NR_154082	NG060671
2	*H. valesiaca*	Roux 2975	France	KU355340	KU355399
3	*Pleurotus abieticola*	HKAS46100	China (Tibet)	KP771695	KP867909
4	*Pleurotus australis*	ICMP 21585	New Zealand: Auckland	MH395977	MH396002
5	*P. australis*	ICMP 18149	New Zealand: Karekare	MH395972	MH395996
6	*P. australis*	RV95/568	Australia	AY315762	AF261432

7	*Pleurotus calyptratus*	CBS 325.85	China	EU424283	EU365640
8	*P. cornucopiae*	CBS 283.37	-	MH855911	MH867415
9	*P. cystidiosus*	CBS 615.80	-	EU424279	EU365636
10	*P. cystidiosus*	ACCC51280	-	EU424280	EU365637
11	*P. cystidiosus* var. *formosensis*	CBS 100129^{T}	Taiwan	NR_103594	NG_057793
12	***P. cystidiosus****	**AMH 10819**	**India**	**PX130393**	**PX130394**
13	*Pleurotus djamor*	CBS 100134	-	EU424287	EU365644
14	*P. dryinus*	CBS 481.72	Germany	EU424292	MH872241
15	*P. eryngii var. tuoliensis*	CCMSSC01433	China	KP867912	KP867900
16	*Pleurotus giganteus*	CMU54-1	Thailand	JQ724360	JQ724361
17	*Pleurotus opuntiae*	SAF 251	Italy	MH620771	MK182780
18	*P. opuntiae*	SAF 252	Italy	MH620772	MK182781
19	*P. ostreatus*	TENN 53662^{T}	-	NR_163515	NG_027634
20	*P. ostreatus*	HKAS84903	Germany	KP867913	KP867901
21	*Pleurotus overstrandensis*	PO2^{T}	South Africa	OR101948	OR101952
22	*P. overstrandensis*	PO1	South Africa	OR101949	OR101953
23	*P. overstrandensis*	PO3	South Africa	OR101947	OR101951
24	*P. overstrandensis*	PO4	South Africa	OR101946	OR101950
25	*Pleurotus parsonsiae*	ICMP 18169	New Zealand	MH395975	MH396000
26	*Pleurotus placentodes*	HKAS51745	China	KR827693	KR827695
27	*P. placentodes*	HKAS57781	China	KR827694	KR827696
28	*Pleurotus pulmonarius*	Dai 19968	China	OL437264	OL434413
29	*P. pulmonarius*	HKAS86009	China	KP867918	KP867906
30	*Pleurotus tuber-regium*	CBS 850.95	Nigeria	MH862563	MH874190
31	*P. tuber-regium*	BORHF0648	Malaysia	MH178086	MH178091

(*) The taxon isolated fresh

3. Results

3.1 *Taxonomy*

***Pleurotus cystidiosus* O.K. Mill., Mycologia 61:889 (1969)** (Figs. 1-4).

Substratum/distribution: Growing on the crevice of the living tree trunk of *Madhuca longifolia* (Konig) Macbr. from the premises of Agharkar Research Institute, Pune, Maharashtra, India.

Description of basidiocarp (Sexual Morph):

Marcomorphology: *Basidiocarp*s fleshy, stipitate, 1–2 in numbers, pleurotoid, yellowish white (4A2) when fresh, light orange (5A4) when dried (1A1), flesh white and spongy, 17–25mm thick, spongy, milky white. *Pileus* laterally attached, acentric, stipitate, pileal surface smooth, 250 ×163 mm. *Lamellae* (gill) free, decurrent, fleshy and leathery, surface yellowish white (4A2) when fresh, light orange (5A4) when dried, wedge-shaped, up to 120 mm long, 12 mm wide from the middle. *Stipe* long and cylindrical, with a smooth surface; persistent, yellowish white (4A2) when fresh, becoming light orange (5A4) upon drying. It gradually widens upward along its length, reaching up to 120 mm in length and 25.5 mm in width at the upper portion. *Odour* mushroomy. *Taste* not recorded. *Spore prints* milky white (1A1). *Macrochemical*

reactions; negative in Schaffer's reaction, no reaction with ammonia and hydrochloric acid, and in potassium hydroxide turns dull yellow.

Micromorphology: *Stipitipellis* (stipe trama) *tissues* hyphoid, interwoven, branched, septate, anastomoses, pigmented, hyaline, up to 8.25 µm wide. *Pileipellis* (pileus trama) hyphae branched, septate, interwoven, smooth-walled, light olivaceous to hyaline hyphal tips obtuse, pigmented, light olivaceous, up to 8.21 µm. *Clamp* connections are rarely present; upper pileipellis tissues are dark olivaceous. *Cystidia* abundant, cheilocystidial type, non-fertile, pedicellate, cylindrical to narrowly clavate, multi-guttulate, branched near the base, upper half portion hyaline and guttulate, lower half portion very light olivaceous, guttulate, smooth-walled, hyaline, 23.80–63.22 × 4.15–7.12 µm. *Basidia* bisterigmate to tetrasterigmate, cylindrical to clavate, smooth-walled, pigmented, hyaline, variable in dimension, 47.30–73.60 × 6.00–8.00 µm. *Sterigmata* 2–4 per basidium, straight to curved, sometimes resembling a horn-like or an incisor canine, short to long, variable in dimension, rarely filamentous and flexuous, hyaline, smooth-walled, 4.85 –8.35 × 0.75–1.77 µm. *Basidiospores* cylindrical, elongated, fusoid to ellipsoid to oblong, slightly tapered towards base, smooth-walled, hyaline, variable in dimension, 10.00–15.88 × 3.23–5.69 µm (x = 13.13 × 4.42µm, n = 30) Qm = 2.97, Q = 2.68–3.09.

Asexual Morph: The micromorphology of the asexual stage of the life cycle of *P. cystidiosus* produced *in vitro* was identified as *Antromycopsis macrocarpa Mill., 1969.* The fungus formed abundant coremia (synnemata) on the colony surface in PDA medium. *Hyphae* branched, septate, loose or arranged in parallel bundles or twisted, pigmented, smooth-walled, variable in dimension, up to 6.30 µm wide. *Clamp connections* at the hyphal septa are often. *Dolipore septa* are rarely present. *Synnemata* consisted of a white myceloid cylindrical stipe measuring 201.33–515.90 × 50.15–150.67 µm (length × width). The stipe apex bore a black-headed, shiny (glazed) droplet composed of a dark liquid mass containing a large aggregation of conidia. *Conidiophores* are compactly arranged, forming well-developed synnemata. *Conidia* light olivaceous to olivaceous brown, variable in shape and size, broadly cylindrical, straight to curved, fusoid to broadly ovoid, with a rounded to truncate base and a rounded apex, measuring 10.0–17.98 × 4.6–8.17 µm.

Material examined: INDIA, Maharashtra, Pune, ARI premise (18° 31' 14.4" N, 73° 49' 53.7" E) on crevice of living *Madhuka longifolia* tree trunk, P.N. Singh, 20th June 2025, Ajrekar Mycological Herbarium, (AMH 10819) India; living culture deposited in National Fungal Culture Collection of India (NFCCI-WDCM 932) NFCCI6265.
GenBank numbers: ITS = PX130393, LSU =PX130394.

Culture characteristics: *Colonies* on PDA after 11 days of growth, front white (1A1), slow growing, floccose, reverse yellowish white (4A2), 61 × 61 mm diam. On PCA after 19 days front white (1A1), slow growing, floccose, reverse light yellow (4A4), 62 × 62 mm diam.

Phylogenetic analysis
ITS and LSU rDNA gene regions were used to determine the isolate's phylogenetic placement. A concatenated ITS–LSU dataset comprising 31 sequences representing species of *Pleurotus* and allied genera was analysed. The concatenated alignment file consisted of 1566 columns, 534 distinct patterns, 363 parsimony-informative sites, 78 singleton sites, and 1125 constant sites. ModelFinder selected TIM2+F+I+G4 as the best-fit substitution model based on the Bayesian Information Criterion (BIC).

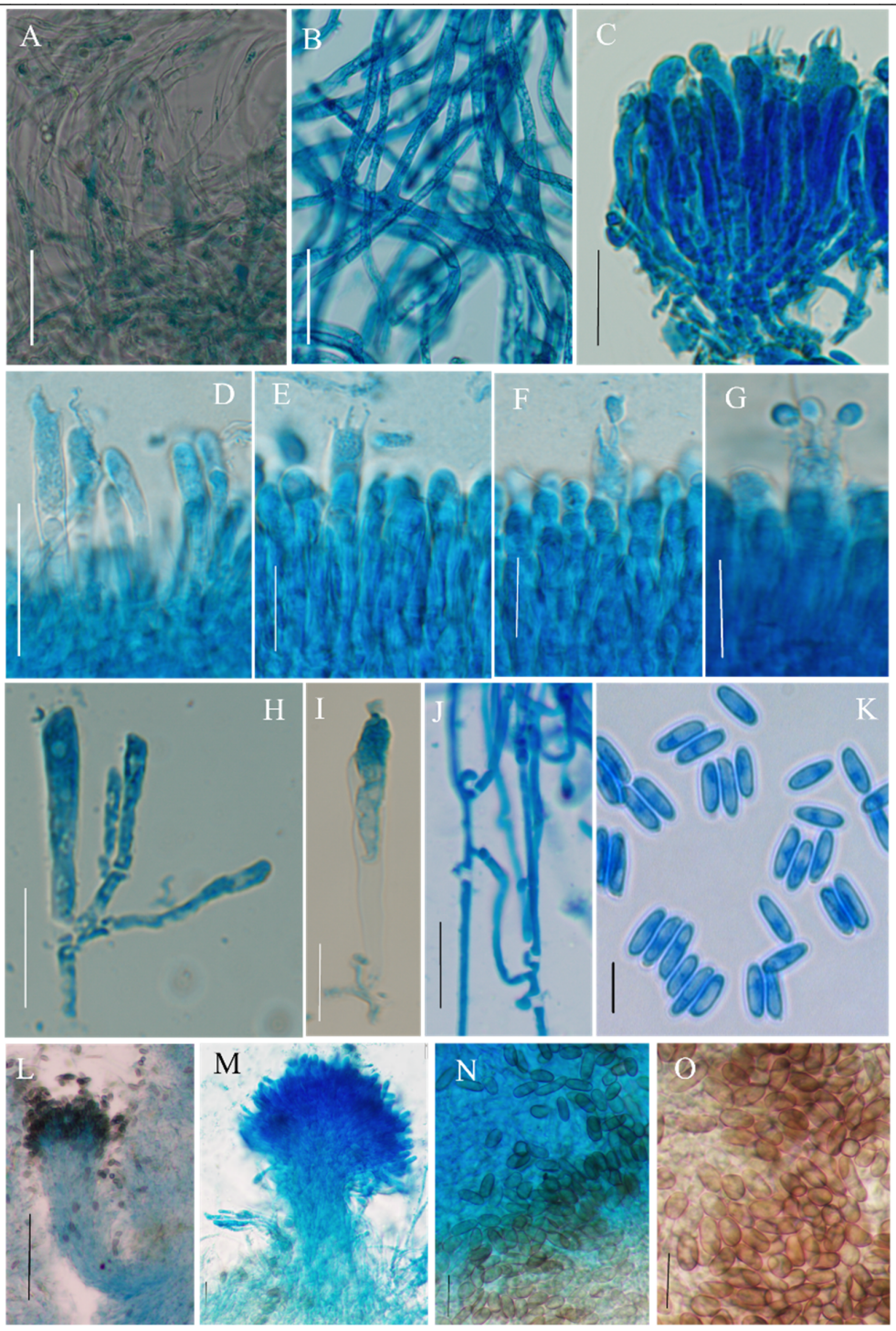

Figure 2. *Pleurotus cystidiosus* (AMH 10819). A. Hyphoid stipe tissues, B. Hyphoid pigmented pileal tissues, C. Basidia with sterigmata and numerous sterile cystidia, D–E Basidia with filamentous type sterigmata, F–G Basidia bearing basidiospores and numerous sterile cystidia, H–I Branched and unbranched cystidium, J. Anastomosed hyphae with clamp connections (in vitro culture), K. Numerous Basidiospores, L–O. Coremia (or synnemata) of a sexual morph (*Antromycopsis macrocarpa*) and numerous conidia (in vitro culture), Scale bars: A–C = 20 µm, D–G=10 µm, H–J = 20 µm, K=10 µm, L= 100 µm, M–O = 20 µm.

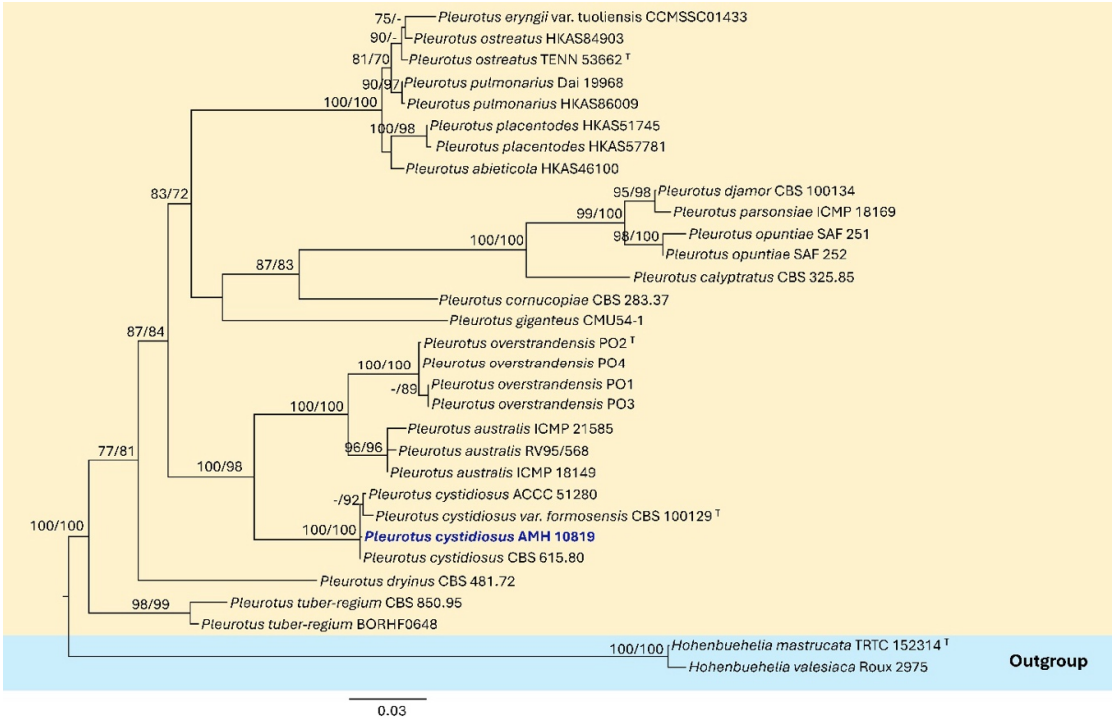

Figure 4. Molecular phylogenetic analysis of *Pleurotus cystidiosus* (AMH 10819), generated by maximum-likelihood (ML) method based on combined ITS, and LSU rDNA sequence data. The taxon isolated in this study is shown in bold blue.

Maximum Likelihood (ML) phylogenetic inference was performed under this model with the following rate parameters: A–C = 2.15807, A–G = 3.82205, A–T = 2.15807, C–G = 1.00000, C–T = 8.05644, and G–T = 1.00000. Base frequencies were estimated as A = 0.260, C = 0.201, G = 0.265, and T = 0.274. The proportion of invariable sites (I) was 0.492, and the gamma shape parameter (α) was 0.515. The log-likelihood value of the consensus tree was −6825.357 (Figure 4). Branch support was assessed using 1,000 ultrafast bootstrap replicates (UFBoot) and the SH-like approximate likelihood ratio test (SH-aLRT) with 1,000 replicates. The combined ITS–LSU phylogeny placed the studied isolate within a distinct and well-supported clade of the genus *Pleurotus*, forming a sister relationship with *P. cystidiosus* CBS 615.80, supported by high SH-aLRT and UFBoot values. Based on concordant morphological characteristics and robust multi-locus phylogenetic evidence, the isolate is herein identified and documented as *P. cystidiosus*.

Discussion: Although *Pleurotus cystidiosus* has previously been reported from India, comprehensive studies integrating both morphological and molecular approaches remain limited. In the present study, the species was re-documented through detailed macroscopic and microscopic characterization along with molecular phylogenetic analyses. Interestingly, the basidiocarps consistently emerged from the same crevice on a living tree trunk, indicating possible substrate specificity and adaptation to a distinct ecological niche. The specimen was successfully isolated in pure culture and identified using a maximum-likelihood (ML) phylogenetic analysis based on combined ITS and LSU rDNA datasets, confirming its placement within the genus *Pleurotus*. The present taxon's asexual morph (10.0–17.98 × 4.6–8.17 µm) shows similarity in the dimensions, shape, and size with the arthrospores/conidia described earlier by Stalpers *et al.* (1990) for *Antromycopsis macrocarpa* (10–15(–18) × 6–7.5(–8) µm. In

addition, the present taxon produced abundant cystidia and a black-headed asexual coremioid stage, as described earlier for the type (Miller 1969). This integrative approach provides a reliable framework for accurate taxonomic identification, particularly for morphologically complex agaricomycetes. The study serves as a useful reference for young and budding researchers, demonstrating how classical morphology can be effectively complemented by molecular tools. Moreover, the re-documentation of this edible and economically important species contributes to a clearer understanding of Indian fungal diversity and underscores the importance of systematically documenting and characterizing indigenous fungal resources for ecological and biotechnological studies.

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