

First Record of *Penicillium clavariiformis* Solms. on Seeds of *Diospyros ferrea* (Willd.) Bakh. from the Northern Western Ghats, India

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Abstract

Penicillioopsis clavariiformis Solms. is a rare filamentous fungus previously reported from various woody substrates and decaying plant matter. In this study, we report the first time host association of *P. clavariiformis* on seeds of *Diospyros ferrea* (Willd.) Bakh., collected from the Manoli Reserve Forest in the Northern Western Ghats, India. The fungus was isolated using standard seed mycobiota screening methods and identified based on both morphological features such as distinctive clavarioid ascomata, septate conidiophores, and cyanophilic conidia and molecular characterization of the internal transcribed spacer (ITS) rDNA region. The ITS sequence showed > 99% similarity with authenticated *P. clavariiformis* sequences in GenBank. This is the first confirmed report of *P. clavariiformis* from seeds of *Diospyros ferrea*, expanding its known ecological and substrate range. The finding highlights the underexplored fungal diversity associated with forest seeds and emphasizes the need for targeted mycological surveys in biodiversity hotspots like the Western Ghats. Our results contribute to the growing knowledge of seed-associated fungi and provide a baseline for future ecological and pathological investigations involving this species.

1. Introduction

Penicillioopsis clavariiformis is a distinctive ascomycete fungus known for its unusual clavarioid ascomata and notable ecological adaptations. Initially reported from the fruits and seeds of *Diospyros paniculata* in Kerala, India, its identification has been confirmed through detailed morphological and molecular analyses (Krishnapriya et al., 2020). A rare halophilic species, *P. clavariiformis* demonstrates a high tolerance to saline conditions, thriving in environments with up to 10% NaCl and showing optimal growth at 24°C (Kashyap et al., 2016). This salt tolerance is linked to its ability to shift in carbon metabolism from glycolysis to the hexose monophosphate (HMP) shunt serves as an adaptive mechanism that maintains cellular function during stress conditions. Enhanced expression of stress-related genes (Hsp98, Hsp60, RHO, HTB) further supports its halotolerance. Morphologically, it is characterized by biverticillate penicilli and oval to ellipsoidal conidia, as observed through scanning electron microscopy. The species was first discovered in a sacred grove, reflecting its niche in ecologically rich and often underexplored habitats (Kashyap et al., 2016).

The genus *Penicillioopsis*, belonging to the family Trichocomaceae, is a tropical group of fungi recognized for its distinct synnemata and varied conidial morphology, including cylindrical to clavate phialides and a range of spore shapes. Although currently comprising a small number of described species, some members, such as *P. clavariiformis*, are notable for producing bioactive mycotoxins with toxicological relevance and potential therapeutic applications, including anti-HIV properties (Douanla-Meli & Langer, 2007). *P. clavariiformis* has been found on fruits and seeds of *Diospyros* species, including *D. paniculata* and *D. crumenata*, and is associated with seed rot disease, causing significant ecological and economic impact. The fungal pathogen *Penicillioopsis clavariiformis* causes significant seed damage in *Diospyros crumenata*, a critically endangered tree with high timber and nutritional fruit value, highlighting the need for holistic seed disease management (Praveena et al., 2025).

Diospyros ferrea, a medium-sized evergreen tree from the family Ebenaceae, is widely distributed across tropical and warm temperate regions, including the Western Ghats, coastal areas, the Deccan Plateau, and dry evergreen forests of India. Known locally in Tamil as “Kattu Thuvurai” and “Irumbuli,” it has traditionally been used as a famine food (Serra et al., 2024). The genus *Diospyros* is highly importance for its dense ebony wood, used usually in high-end furniture and musical tools, and for its medicinal properties. Bioactive compounds such as naphthoquinones and triterpenoids found in these species have shown promise in treating infectious diseases (Meeprom et al., 2024; Ribeiro et al., 2023). Despite its economic and ecological importance, *D. ferrea* faces threats from fungal pathogens, particularly cercosporoid ascomycetes like *Pseudocercospora* spp., which exhibit cryptic speciation and complicate disease management (Braun et al., 2020).

Diospyros ferrea differs from other *Diospyros* species by its evergreen habit, thick leathery glossy leaves, very hard and dense wood, smooth grey-black bark, and larger globose to ovoid fruits with persistent calyx lobes (Serra et al., 2024). It holds notable phytochemical and medicinal significance. Traditionally, its roots have been used to treat ailments such as dyspepsia and inflammation, a practice supported by the presence of bioactive compounds identified through various solvent extraction techniques. Studies have revealed that ethanol extracts contain the highest concentration of phytochemicals, including flavonoids, terpenoids, alkaloids, and tannins, underscoring the importance of selecting appropriate solvents for maximizing extraction efficiency (Vijayalakshmi & Ravindhran, 2012). Medicinally, leaf extracts of *D. ferrea* exhibit strong anti-inflammatory, antibacterial, and antioxidant properties, aligning with their traditional use in wound healing (Khumaidi et al., 2024). Additionally, several *Diospyros* species have demonstrated anticancer potential, with compounds shown to inhibit tumor progress and promote programmed cell death in cancerous cells (Rauf et al., 2024). While these findings highlight the therapeutic promise of *D. ferrea*. The study of *P. clavariiformis* not only underscores its unique physiological traits but also highlights the need for continued exploration of fungal biodiversity, particularly in extreme or specialized ecosystems where novel species and adaptations are likely to be found.

2. Materials and Methods

2.1. Field Collection and Fungal Isolation

Fruit body samples of *Penicillliopsis clavariiformis* were collected from the fruits and seeds of *Diospyros ferrea* during October 2021 and September–October 2024 in the Manoli Reserve Forest, Maharashtra, India (16.92296°N, 73.79556°E). Geographical locations have been marked in Fig. 1. The samples were transported to the Research Laboratory of Mycology and Plant Pathology at Shivaji University, Kolhapur, in sterile plastic bags. The specimens were surface-sterilized by method of Petrini et al. (1982). Infected tissue sections from the fruits and seeds were aseptically blotted dry, cut into tiny pieces, and inoculated onto PDA (Potato Dextrose Agar) enriched with streptomycin. The cultures were incubated at $28 \pm 2^{\circ}\text{C}$ for a period of 8 days, following which pure fungal colonies were isolated and stored at 4°C for further examination.

To ensure compliance with good taxonomic practice, a voucher specimen of the host plant (*Diospyros ferrea*) has been deposited in the SUK Herbarium (Shivaji University, Kolhapur, MH, India) under voucher number ABM006. The fungal voucher specimen of *Penicillioopsis clavariiformis* obtained from seeds of *Diospyros ferrea* has been deposited in the Agharkar Research Institute, Mycology and Plant Pathology Department, Pune, MH, India under accession number AMH: 10820. All collection work in this study complied with relevant institutional, national, and international guidelines and legislation. Prior permission was obtained from the Maharashtra State Biodiversity Board (MSBB/Desktop-5/Research/Permission/640/23–24), Govt. of Maharashtra, India for the collection of *Diospyros ferrea* seeds.

2.2. Microscopic Examination:

Microscopic features of the fungal isolates were examined using cotton blue and lactophenol-stained preparations. Freshly mounted slides from pure cultures were observed under a LEICA DM2000 compound microscope at 1000× magnification, and images were captured for documentation. Spores were measured to determine their dimensions, including the length, width, and the spore quotient (Q), which is the length-to-width ratio. The average spore quotient (Q_m) was also calculated.

2.3. Genomic DNA Isolation and ITS Region Amplification:

Genomic DNA was isolated from a fungal culture designated as sample CL using standard CTAB-based methods (Kim et al., 2010). PCR amplification was carried out in a 25 µL reaction volume containing approximately 20 ng of genomic DNA, using fungal-specific primers ITS1 and ITS4 to target the internal transcribed spacer (ITS) region (White et al., 1990). The amplified PCR products were resolved using a 1.5% agarose gel electrophoresis set in 1.0% TBE buffer to verify successful amplification.

2.4. Sequencing and Species Confirmation:

Amplified PCR products were purified using a commercial DNA purification kit and subsequently sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit. Sequencing was performed on an Applied Biosystems ABI3500 DNA Sequencer to generate high-quality nucleotide data. The obtained ITS sequence was analyzed for quality and compared with reference sequences using the BLAST algorithm against the NCBI GenBank database. A phylogenetic tree was constructed based on the ITS sequence data to determine the evolutionary relationship of the identified fungus with related taxa.

The evolutionary relationships among 34 fungal taxa were inferred using the Neighbor-Joining (NJ) method as described by Saitou and Nei (1987). The optimal phylogenetic tree obtained had a total branch length of 0.302. The bootstrap test with 1,000 replicates was performed to assess the statistical support for the clustering of taxa; bootstrap values are shown above the branches (Felsenstein, 1985). The evolutionary distances between sequences were calculated using the Maximum Composite Likelihood (MCL) method, and the results are expressed as the number of base substitutions per site

(Tamura et al., 2004). The dataset comprised 34 nucleotide sequences, and the pairwise deletion option was applied to handle ambiguous positions, resulting in a final alignment of 1,830 positions.

All evolutionary analyses were conducted in MEGA version 12 (Kumar et al., 2024), utilizing up to three parallel computing threads for enhanced computational efficiency.

3. Results

3.1. Taxonomy:

The fruiting bodies reached heights of up to 6–9 cm and measured 1–3 mm in width (As shown in Fig. 2i). They appeared upright to slightly curved and ranged from simple, unbranched forms to occasionally branched structures (Fig. 2). In branched specimens, terminal branches display irregular patterns. The structures tapered towards the apex, were smooth-surfaced, and solid rather than fragile. They emitted an unpleasant odor, and the spore print was distinctly yellow. Fresh specimens exhibited a vivid yellow coloration, which faded to a dull brown upon drying.

The conidiophores measured between 6–7 μm in width and were pale brown. They exhibited distinct ring-like septations, with each segment often swollen at both ends. Their surface looks smooth, and the walls were moderately thick, about 0.5–1 μm . Short branches (ramuli), arising in groups of conidiophores. Metulae hyaline to pale brown. Phialides were cylindrical and hyaline. Conidia ($n = 20$) ranged in size from 6–8 $\times$ 4–5 μm , with a Q-value of 1.5–1.6 and a mean Q_m of 1.55. They were sub globose to ellipsoid, eguttulate, hyaline, densely granular, and had smooth to slightly ornamented surfaces. Conidial walls were about 1 μm thick and showed cyanophilic reactions in cotton blue (Fig. 3).

3.2. Molecular profiling and phylogenetic analysis:

Sequencing of the ITS region from the collected specimen (CL) produced a 498 base pair fragment. A BLAST search of the ITS sequence against the NCBI GenBank database showed 99.40% identity with existing *Penicillioopsis clavariiformis* strains. The sequence labeled as *Penicillioopsis* (novel isolate) clusters closely with reference sequences PP218122.1 and MK459552.1, forming a well-supported clade with a bootstrap value of 100, confirming its taxonomic placement within the *Penicillioopsis* genus. This clade is further supported by proximity to other related fungal accessions, including EF567419.1 and LC830717.1. The upper part of the tree includes multiple accessions from diverse genera, including KY401087.1, MN543977.1, and NR158824.1, which form separate clusters with varying bootstrap support, reflecting their evolutionary divergence. The basal branch, including EF669714.1 and MN431401.1, may serve as an outgroup, anchoring the tree and assisting in rooting the evolutionary relationships (Fig. 4).

4. Discussion

Species of the genus *Penicillioopsis* Solms are predominantly found in tropical regions and are commonly associated with seeds instead of soil. As of 2024, the genus comprises seventeen recognized species (Praveena et al., 2025). *Penicillioopsis clavariiformis* has been reported from various *Diospyros* hosts, including *D. crassiflora* (Douanla-Meli & Langer, 2007), and *D. macrophylla* (Solms-Laubach, 1887), *D. crumenata* (Praveena et al., 2025), while, occurrence of *Penicillioopsis pseudocordyceps*, a newly identified species from *Diospyros discolor* seeds was reported by Hsieh and Ju (2002). There are no confirmed reports of *Penicillioopsis clavariiformis* occurring on host plants outside the genus *Diospyros*. All documented occurrences so far, indicate a host-specific association. This suggests a potential co-evolutionary relationship or ecological specialization, though more research is needed to verify whether this exclusivity is due to host preference.

These fungi exhibit a specialized ecological preference for seeds, and are infrequently encountered in soil, suggesting a narrow ecological niche. Phylogenetically, *Penicillioopsis* shows affinity with genera such as *Talaromyces* and *Penicillium* but can be distinguished by features like stipitate ascomata and unique reproductive structures. However, comprehensive molecular studies remain limited, highlighting the need for further phylogenetic analysis to clarify relationships within the group (Douanla-Meli & Langer, 2007).

The report of *Penicillioopsis clavariiformis* from *Diospyros paniculata* (Krishnapriya et al. 2020), extends the known host range of this species, which appears to have a specific ecological association with members of the genus *Diospyros*. Morphological and molecular comparisons confirm its distinction from related taxa such as *P. africana* and *P. bambusae*, based on synnemata color, conidial dimensions, and host specificity. The observed yellow pigmentation, attributed to penicilliopsin, along with its halotolerant properties, supports earlier findings on its ecological adaptability and potential bioactivity (Krishnapriya et al. 2020; Douanla-Meli & Langer, 2007). While *Penicillioopsis* is within the Eurotiomycetes (family Aspergillaceae), most clavarioid fungi belong to different taxonomic groups, such as Basidiomycota (e.g., Clavariaceae). This places *Penicillioopsis* as a morphologically convergent but phylogenetically distinct group compared to typical clavarioid fungi (Desjardin, 2015; Krishnapriya et al. 2020).

The present specimen shares key morphological traits with *P. clavariiformis* from *Diospyros paniculata* (Krishnapriya et al. 2020), such as pale brown conidiophores with distinct septations, cylindrical hyaline phialides, and subglobose to ellipsoid, cyanophilic conidia with granular contents. These features align closely with those reported from Kerala. Compared to *P. Africana* (Samson & Seifert, 1986), which also has stipitate stromata and verticillate conidiophores, the absence of tentaculate synnemata and larger conidia in the present sample help distinguish it. This record of *Penicillioopsis clavariiformis* on *Diospyros ferrea* expands its known host range within the *Diospyros* genus and highlights its potential role as a specialized, seed-inhabiting pathogen. Its consistent association with seed tissues across multiple hosts suggests ecological adaptability and possible implications for seed viability and regeneration. Given the ecological importance of *Diospyros* species in the Western Ghats, this finding underscores the need for

continued seed health monitoring and further investigation into the biology, host specificity, and ecological impact of *P. clavariiformis*.

5. Conclusion

This study documents the first confirmed occurrence of *Penicillium clavariiformis* on the seeds of *Diospyros ferrea* from the Manoli Reserve Forests in the Northern Western Ghats of India. Identification was based on a combination of distinctive morphological characteristics—such as clavarioid ascomata, ring-septate conidiophores, and cyanophilic conidia—and molecular analysis of the ITS region, which revealed a 99.40% similarity to authenticated *P. clavariiformis* sequences. The phylogenetic analysis confirmed the placement of the novel *Penicillium* isolate within its genus, supported by high bootstrap values and close clustering with reference sequences. This finding expands the known host range of *P. clavariiformis* within the genus *Diospyros* and suggests a possible host-specific or seed-associated ecological relationship. The results highlight the value of integrating morphological and molecular approaches in fungal biodiversity assessments and underscore the need for further investigations into the pathogenic potential, host specificity, and ecological functions of *P. clavariiformis*. Continued exploration of underdocumented habitats such as the Western Ghats remains critical for uncovering fungal diversity and informing conservation and seed health management strategies.

Declarations

Ethical Approval: Not applicable

Consent to participate: Not applicable

Consent to publish: Not applicable

Data Availability Statement:

The datasets generated and/or analysed during the current study are available in the NCBI GenBank repository, <https://www.ncbi.nlm.nih.gov/nuccore/PX136227.1?report=GenBank>, under accession number PX136227.

Authors Contributions:

PB and AM: collection, methodology, investigation, data curation, formal analysis, and writing-original draft. KS: data curation, and formal analysis. SK: collection, data curation, and formal analysis. MB and SS: supervision, investigation support, validation, and resources. ShivajiK: supervision and validation. MN: supervision, investigation, resources, writing- review & editing.

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Competing Interests: Not applicable

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Figures

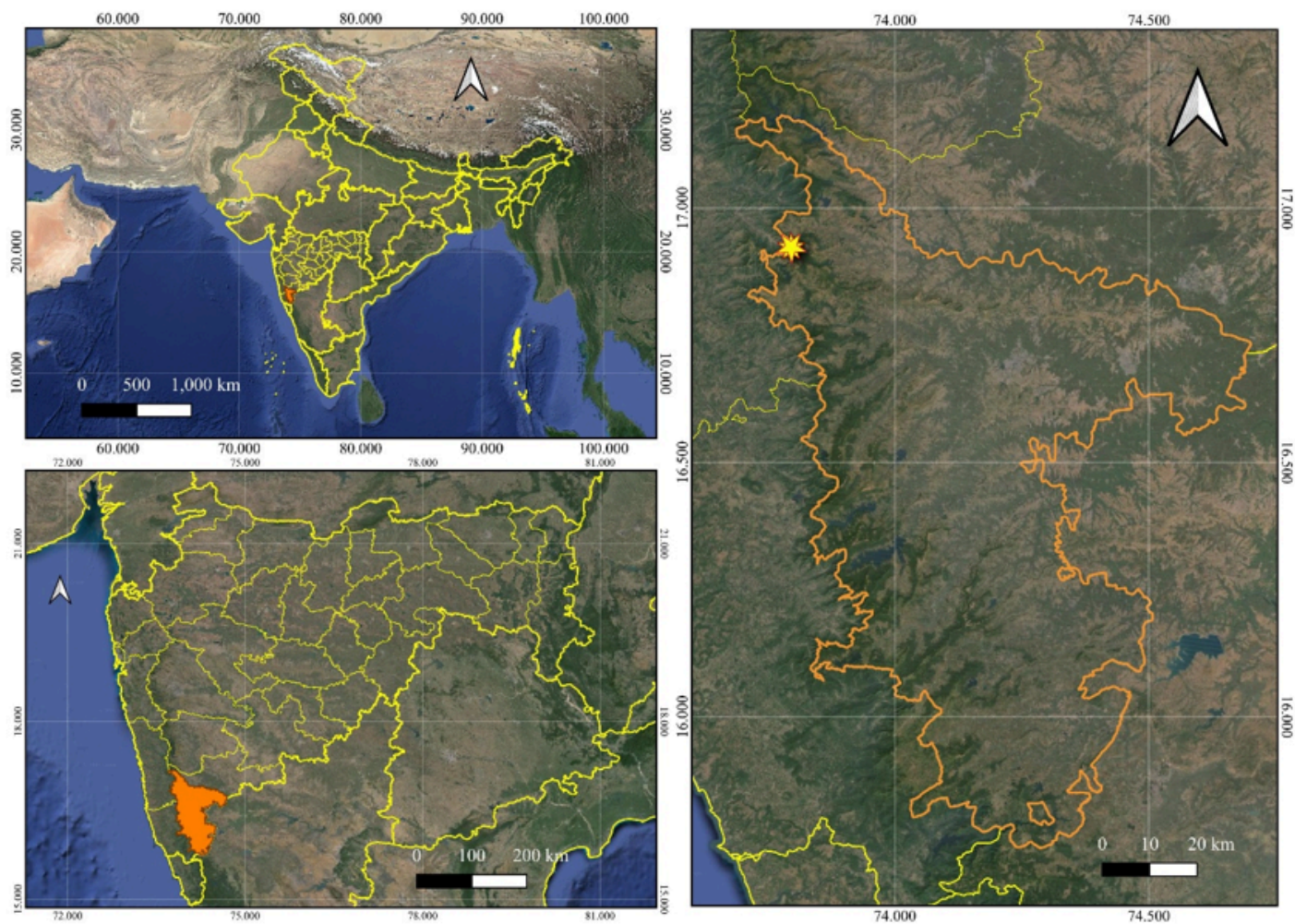


Figure 1

Geographical map showing the collection site of *Penicillioopsis clavariiformis* from the seed samples of *Diospyros ferrea* in the Northern Western Ghats, Maharashtra, India.

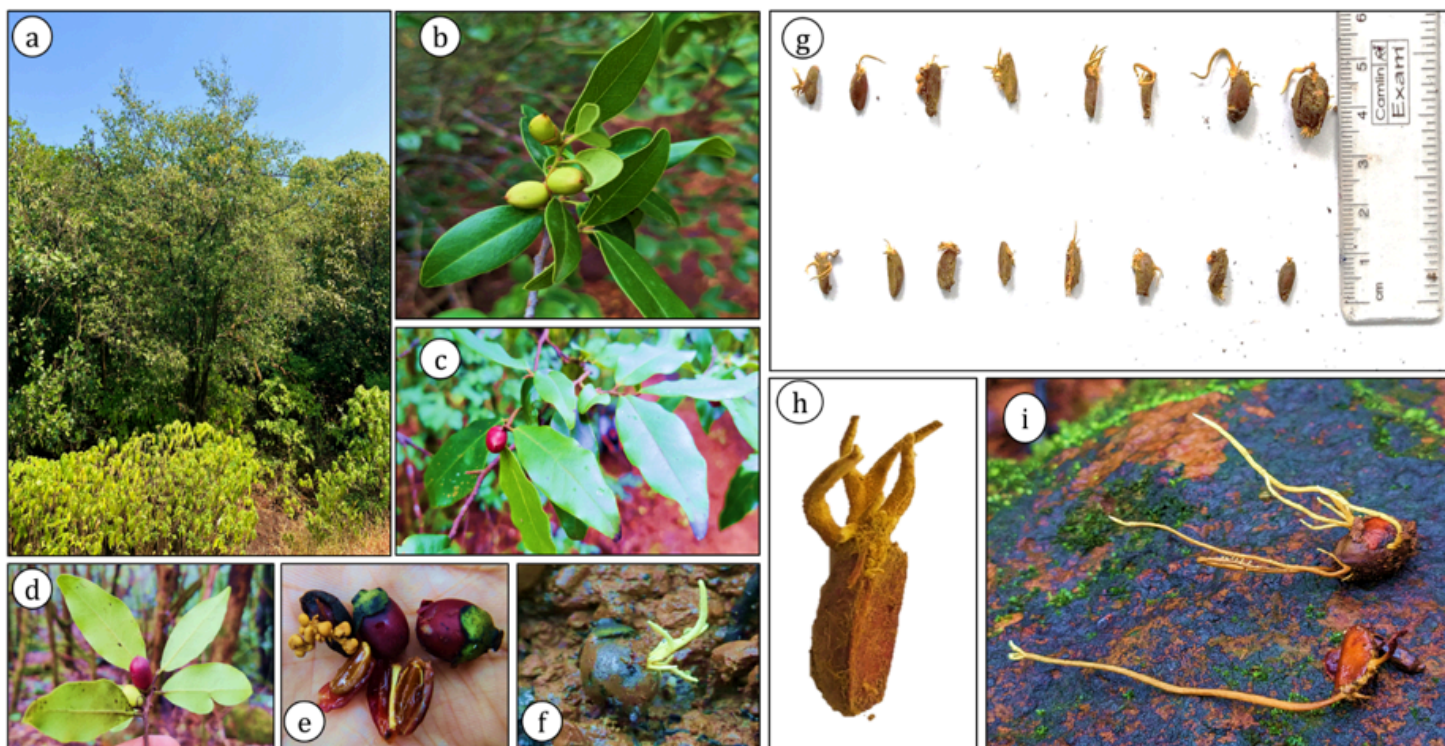


Figure 2

a. A habitat of *Diospyros ferrea*; b-d. Fruits of *D. ferrea*; e-i. Ascomata of *Penicillium clavariiformis* on Fruit and Seeds of *D. ferrea*.

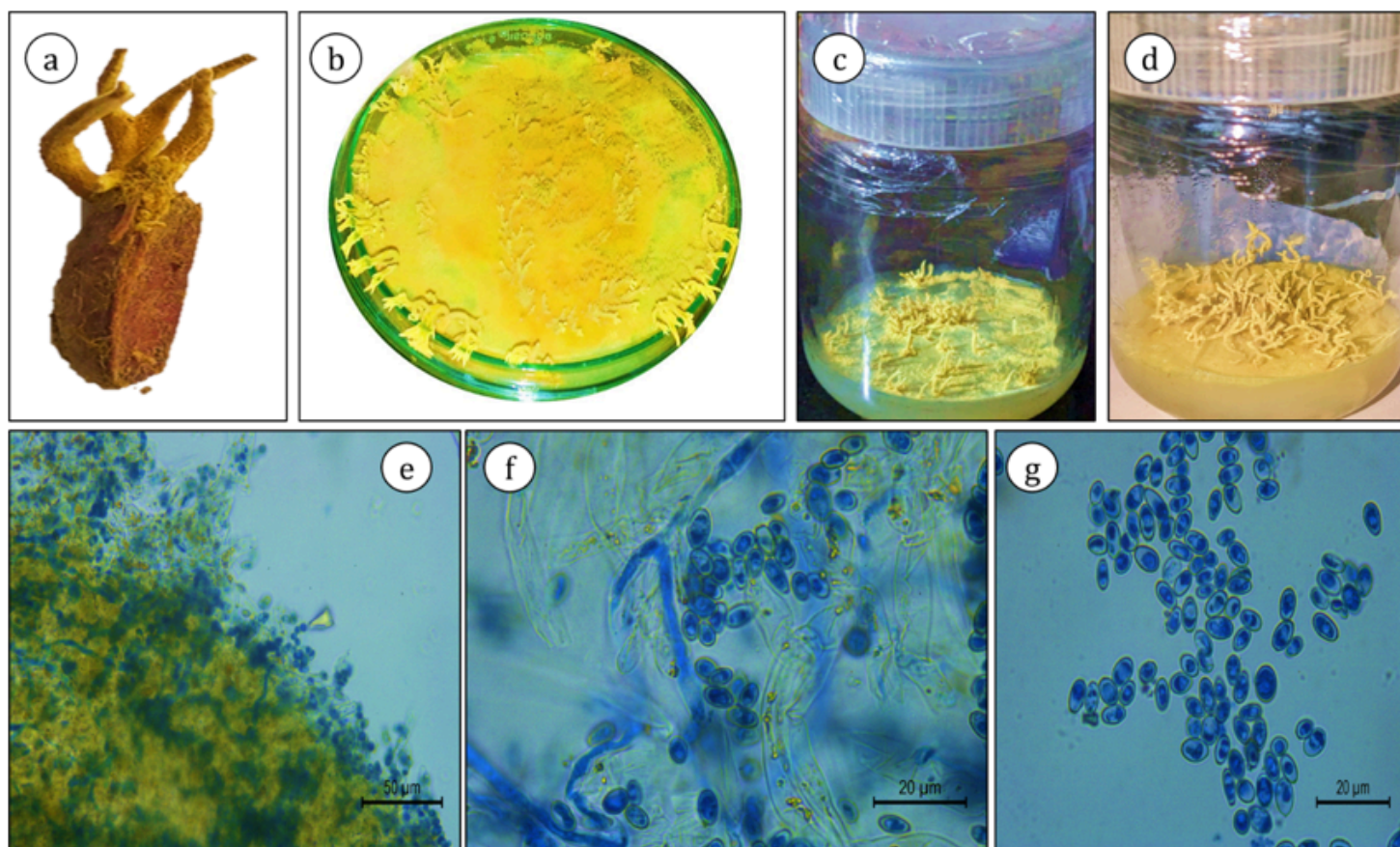


Figure 3

a. *Penicillium clavariiformis* on *D. ferrea*; b-d. Pure culture of *P. clavariiformis*; e-f. The conidiophore of *P. clavariiformis* exhibits characteristic ramuli, metulae, and cylindrical phialides; g. Conidia of *P. clavariiformis*.

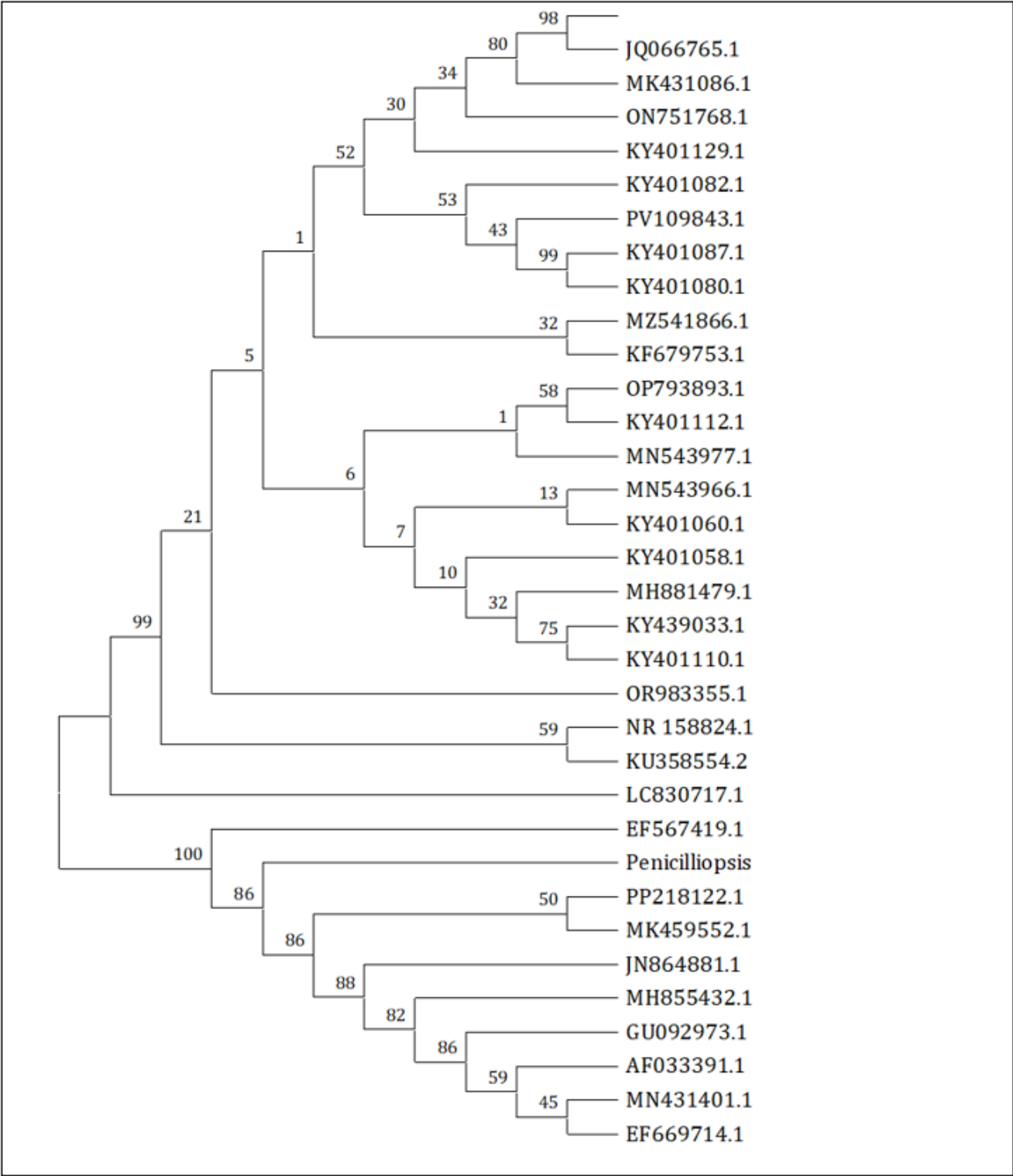


Figure 4

Phylogenetic tree constructed using the Neighbor-Joining method based on ITS rDNA sequences, showing the evolutionary relationship of *Penicillioopsis clavariiformis* with related fungal taxa. Bootstrap values (1000 replicates) are shown at the branch nodes. The tree was generated using MEGA12 (Version 12.0.11) with default settings and rooted using EF669714.1 as the outgroup.