Isolation of Taxol and Flavin-like fluorochrome from Endophytic Fungi of *Mangifera indica*

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Abstract

Scouting for novel and plant derived biomolecules from endophytic microbial sources draws greater focus on the discovery of novel bioactive metabolites. With this rationale, we scouted the endophytic fungi for taxol, an anticancer diterpenoid and fluorescent biomolecules. In the present study, about 31 endophytic fungal isolates recovered from the *Mangifera indica* leaves were screened for taxol production in M1D medium. About five isolates were short listed based on the thin layer chromatographic analysis of the fungal extracts. Among them *Colletotrichum* sp. MIP-5 has been identified as a producer of fungal taxol based on UV, FTIR, TLC and HPLC analysis. The partially purified fungal taxol showed similar spectral and chromatographic features of commercially available paclitaxel. In addition to this, we also report the production of a fluorescent compound by *Penicillium* sp. MIP-3. The Flavin-like compound exhibited a bright greenish yellow fluorescence with an emission maximum in the range of 505 – 545nm. GC-MS analysis showed the occurrence of *Latia* luciferin, primarily associated with the bioluminescence of freshwater limpet *Latia neritoides*. This is the first report of this compound from *Penicillium* sp. In addition, therapeutically active steroid (β-Sitosterol, Stigmasterol, Campesterol), quinones (Benzo[h]quinoline, 2,4-dimethyl-) and phloroglucinol (Aspidinol) derivatives were also identified from *Penicillium* sp. MIP-3 based on GC-MS analysis. These molecules could potentially be used in biological and pharmaceutical applications in future.

Keywords: Bioprospecting, Taxol, Luciferin, Fluorescence, anticancer
INTRODUCTION

Endophytic fungi, an enormously diverse group, habituating the living internal tissues of plants without causing any immediate overt negative effects, represent a rich source of novel natural products.1 Interactions of these cryptic organisms with their host plants vary coherently from mutualism to parasitism depending on the refined balance between the demands of the invaders and the host response.2 In addition, the multitude of interactions among endophytic fungal partners and other biotic components of the niche yields a repertoire of diverse secondary metabolites.3 The biological activity of these metabolites range from biosynthesis of defence compounds (antimicrobial, insecticidal, etc.,) which can exert activity directly or trigger (precursors) / modulate (epigenetic modulators) the hosts defence pathways to confer host benefits (fitness and protection).4 Also, they were documented to modulate the host microbiome through quorum sensing5,6 and confer stress mitigation through host adapted endophytic fungal symbiosis.4 Considerable interest on bioprospecting them for wider applications such as agricultural, industrial and pharmaceutical has been well documented.7,9

Endophytic fungi represent a treasure trove of diverse metabolites and earns special attention as a source of novel bioactive compounds of immense therapeutic applications.10-12 They were also reported to produce plant mimetic metabolites and novel structural metabolites.13,14 Discovery of the diterpenoid anticancer drug paclitaxel (Taxol), from an endophytic fungus (Taxomyces andreanae) of Pacific Yew (Taxus brevifolia)15 had drawn greater attentions to the hidden endophytic fungi as a potential source of therapeutic biomolecules.9,16-18 Elaborate reviews summarizing the chemical (alkaloids, flavonoids, fatty acids, sterols, glycosides, poly heterocyclic compounds, small peptide conjugates, etc.)19-22 and functional (antioxidant, anticancer, antimicrobial, antiviral, etc.)22-26 diversities of the endophytic fungal metabolites have enriched the knowledge about these novelties and aids in developing new drugs based on their pharmacophores.3,27 Studies on bioluminescent28,29 and fluorescent30-32 fungal metabolites were relatively few and unravelling them offers a potential scope for furthering the real-time in vivo monitoring of biological functions in living systems.33-35

Fungi not only manifest diverse metabolic profile, but also exhibit pleomorphic and phenotypic plasticity in nature.36,37 Owing to these phenomenon and subjectivity associated with the description of morphological features employed in the identification processes, molecular identification methods were widely embraced to provide much more robust solution for taxonomic queries. ITS-based molecular identification continue to be in practice for about three decades and it’s been reviewed in length.38,39 The vastness of the fungal ITS data in public domains encompassing most of the documented taxa also renders an excellent opportunity to obtain a well-curated reference dataset against which query sequences could be compared.39

In this study, endophytic fungal flora from Mangifera indica, a perennial horticultural tree with excellent nutritive and medicinal values, was subjected to investigation in this study. Earlier reports have suggested the presence of diverse bioactive metabolites82 and vibrant endophytic fungal community83 in the M. indica. Investigations on the bioactive metabolites of these fungal communities were minimalistic and none could be attributed to taxol related metabolites, hence the endophytic fungi recovered from mango leaves were screened for production of taxol, a diterpenoid anticancer metabolite. The Serendipitous observation of fluorescence in the culture filtrate of one of our fungal isolates motivated to explore them further. The potential taxol producers and fluorescence emitting isolates were identified based on phylogenetic analysis of ITS sequences.

MATERIALS AND METHODS

Plant Collection and Surface sterilization

Healthy (Asymptotic) and mature leaves of Mangifera indica were collected from Madurai Kamaraj University campus; in sterile polythene bags and transported carefully to the laboratory. The leaf samples were subjected to a surface sterilization procedure which involves initial wash in running tap water to remove soil particles and adhered debris, and subsequently washed with distilled water. This was followed by washing in
70% ethanol for 30 seconds, later soaked in 4% sodium hypochlorite for 90 seconds and finally, the leaves were rinsed thrice in sterile distilled water. Thoroughly surface sterilized leaves were chopped into small uniformed squares using a sterile scalpel. The leaf segments were inoculated into the PDA media under aseptic condition and incubated in growth chamber. After 72hrs of incubation in 12hr/12hr:light/dark cycle at 25°C, fungal hyphae emerging from leaf segments were transferred to new PDA plates for establishing their axenic culture. A control PDA plate with imprints of surface sterilized leaf segments were maintained all along the study to test the efficacy of surface sterilization protocol.

Secondary metabolites production

Seven days old fungal biomass was cut from the agar plate in to 1cm² of area and was aseptically transferred to the 100ml of sterile M1D media prepared in 250ml of conical flask for secondary metabolite production. The inoculated flasks were incubated in static condition for 21 days at 25°C (± 2°C) with 12 h light/12 h dark cycle. After 21 days of incubation, the fungal culture fluid was filtered through three layers of filter paper to remove mycelia. The extra cellular fungal compounds in the culture filtrate were extracted with organic solvent Dichloromethane (DCM) in ratio 1:2 V/V (Fungal extract: DCM respectively). The organic phase was collected and the solvent was then removed by evaporation under reduced pressure using rotary vacuum evaporator. A portion of dry solid residue was re-dissolved for bioprospecting studies.

Screening and Characterization of taxol

Thin Layer Chromatography

The fungal metabolites condensates recovered in the previous step was analysed on 1mm silica coated TLC plate (Stationary Phase). TLC jar was properly equilibrated with the solvent system of Chloroform: Methanol (9.2:0.8 V/V) (Mobile Phase), as reported by Chakravarthi et al. for taxol screening. Analytes were carefully spotted as small uniform spot along with the authentic paclitaxel (Sigma Aldrich) on TLC plate and co-developed in the above mentioned solvent system. TLC plate was taken out from the tank, and visualized in UV illuminator. Putative taxol and paclitaxel would fluoresce blue under UV. Corresponding bands were scrapped and eluted with methanol for further analysis. The retention factor ($R_f$) was calculated with the formula, $R_f = \frac{\text{Distance travelled by analyte}}{\text{Distance travelled by solvent front}}$.

Spectral and Chromatographic analysis

Putative taxol samples resuspended in methanol were analysed using UV-Visible spectrophotometry (HITACHI U-2900 spectrophotometer) and Fourier Transform Infra-Red spectroscopy (Shimadzu FT IR 8000). The spectrums were compared with the standard paclitaxel to ascertain the identity. Also the partially purified putative taxol was analysed by high performance liquid chromatography (Shimadzu shim-pack CLC ODS (4.6 mm x 15 mm)), liquid pump-LC-6AD, system controller-SCL-6B, UV–Vis detector (195–700 nm)-SPD6AV, data processor-CR-5A) using mobile phase consisting of methanol:water (65:35) at flow rate of 1 ml/min. Injected sample (20 µl) were analysed at 227nm and the retention time of the recorded peaks were compared against the paclitaxel.

Screening and Characterization of Fluorescent compound

Thin Layer Chromatography

The DCM extracts of three fungal isolates which showed fluorescent bands under UV illumination were re-developed on TLC plate with Chloroform: Methanol (8:2 V/V) (Mobile Phase). The most prominent fluorescent band was scrapped out and eluted with methanol. The analyte was subjected to slow evaporation and subjected to further spectral and chromatographic analysis.

Spectrofluorometric analysis

The partially purified compound was re-dissolved in DCM and subjected to spectrofluorophotometric analysis (Cary Eclipse Fluorescence spectrometer) in synchronous mode (300nm – 700nm) to determine the excitation and emission maxima of the compound.

Solvatochromism

The partially purified compound (10mg) was dissolved separately in eleven solvents (Hexane, Toluene, Dichloromethane, Chloroform, Ethyl acetate, Acetone, Methanol, Acetonitrile, Acetic acid, Dimethyl sulfoxide and water) differing in their polarity index. After gentle mixing, the
mixtures were observed under UV illuminator and fluorescence intensities were monitored using spectrofluorometry.\(^{45}\)

**GC-MS analysis**

The DCM extract of the fungi (MIP-3) with prominent fluorescence was subjected to GC-MS analysis (Agilent GC 7890A / MS5975C) using Agilent DB5MS column of 30m (length) × 0.25mm (internal diameter) × 0.5µm (film thickness) and Helium gas (99.99% pure) at a flow rate of 1 ml/min was used as the carrier gas. For Mass detection, an electron ionization energy method was employed operating at 70 eV (electron Volts) with 0.2 s of scan time and fragments ranging from 40 to 600 m/z. The injection quantity of 1 µL was used (split ratio 5:1), and the injector temperature was maintained at 250°C (constant). The column oven temperature was set at 50°C for 1 min, raised at 12°C per min and final temperature was increased to 300°C for 2 min. The mass fingerprints obtained were matched with the spectral database from the National Institute of Standards and Technology (NIST) library.\(^{46}\)

**Identification of fungal isolates**

The two fungal isolates MIP-3 (producing fluorescent compound) and MIP-5 (potential taxol producer) were subjected to morphological (spore and culture features) and ITS based molecular identification. The mycelial and spore characteristics were recorded under light microscopy (Olympus CX23). Genomic DNA of axenic cultures was extracted using the method described by Cenis\(^{47}\) and was utilized for PCR amplification of ITS region. Universal ITS primers [(ITS1 5’TCCGTAGGTGAACCTGCGG 3’) and (ITS4 5’TCCTCCGCTTATTGATATGC 3’)]\(^{48}\) were used to amplify the internal spacer region spanning the ITS1-5.8S-ITS2 segment. A 25µl reaction mixture comprised of 20ng genomic DNA, 12.5 µl of 2x DNA master mix (Amplicon), 1µl each of the forward and reverse primers (Sigma Aldrich). PCR amplification was performed in Biorad T100 instrument according to the following reaction conditions: 4 min at 94°C for initial denaturation, 30 cycles each of 30 seconds at 94°C for denaturation, 1min at 58.2°C for annealing, 2 min at 72°C for extension, and a final extension at 72°C for 7min. The amplicons were verified on 1.5% agarose gels, purified and sequenced using the same primers at Eurofins Pvt. Ltd, Bangalore, India. Sequences from both the reads were merged to get the full-length sequences as detailed earlier.\(^{49}\) They were blast searched among sequences of type specimens available in NCBI database to identify the closest similar sequences. The closely related sequences were chosen for phylogenetic reconstruction.

![Fig. 1. The culture morphology of 31 endophytic fungal isolates recovered from Mangifera indica leaves.](image-url)
through the maximum likelihood method, with 1000 bootstrap iteration in Mega X. Also, the nucleotide substitution model (Kimura2 + G) best defining the dataset was chosen using the inbuilt options to construct the phylogenetic tree. The generated phylogenetic tree was redrawn using iTOL v6 (https://itol.embl.de/).

RESULTS
Thirty-one endophytic fungal isolates were recovered from the Mangifera indica leaves and were presented in Fig. 1. Axenic cultures were stored and maintained in the lab repository for future studies.

Screening and Characterization of Putative Taxol
Of the 31 isolates grown in M1D medium, on TLC based screening, bands of 5 isolates co-migrated with the authentic paclitaxel at same \( R_f \) (0.40) and were considered to be potential producers of taxanes (Fig. 2). Among the five isolates, MIP-5 isolate exhibited spectral and characteristic features of authentic paclitaxel (Fig. 3). It had absorption maxima at 237nm and 275nm in UV-visible spectroscopy. In FTIR spectrum, a broad peak at 3375-3390 cm\(^{-1}\) showed the presence of OH stretching in the compound, while a peak at 2945 cm\(^{-1}\) revealed the presence of CH stretching and peaks at 2364 and 1454 cm\(^{-1}\) depicts the NH stretching. The COO stretching peaks were observed at 1383 and 671 cm\(^{-1}\) while peaks in the 1114-1028 cm\(^{-1}\) were predominantly due to the presence of aromatic C and H bends. These peaks correspond to the fingerprints of authentic paclitaxel. Also the HPLC data recorded for the putative taxol from MIP-5 isolates had a retention time around 4.4 min and it was in consistence with the data recorded for paclitaxel.

Screening and Characterization of Fluorescent molecules
The DCM extract of MIP-3 isolate fluoresced under UV illumination (Fig. 2) and hence was subjected to further analysis. Maximum production of the fluorescent molecule was observed to be during the 15\(^{th}\) day of growth in M1D medium (Fig. 4). The fluorescent band observed on the TLC plate was scrapped and eluted in methanol. The partially purified fluorescent molecule dissolved in DCM exhibited an excitation maximum at 400nm and emission in the range of 518nm (Fig. 5). The influence of the solvent polarity on the fluorescence was analysed (Fig. 6) and although no direct correlation could be observed, few interesting observations were recorded. A significant loss in the fluorescence intensity and shift in the emission maximum (\( E_m \)) was evidenced when water (polarity index, PI=10.1, \( E_m = 545nm \)) and Methanol (PI=5.1, \( E_m = 538nm \)) were used as the solvent. Although

Fig. 2. Thin layer chromatograms (A) shows the DCM extracts of fungal isolates with putative taxol bands. PT denotes the paclitaxel (Sigma-Aldrich). MIP denotes the fungal extracts from different isolates. (B) shows the fluorescent bands in the DCM extracts of fungal isolates (MIP-3 and MIP-20)
the fluorescent intensity dropped when Hexane (PI=0.1, $E_m = 509nm$) was used as the solvent, $E_m$ was observed to be well within the range of 500-520nm, exhibited by other solvents used in this study.

GC-MS analysis of the DCM extract of MIP-3 revealed the presence of 1-Buten-1-ol, 2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, formate, (1E)-, also known as Latia luciferin. In addition, steroids and steroid derivatives constituted to the majority of the compounds identified in the study (Table 1). β-Sitosterol accounted for (44.79%), Aspidinol (21.8%), Stigmasterol (9.33%), Campesterol (5.4%), p-Butyrophenetidide (3.05%), Benzo[h]quinoline, 2,4-dimethyl- (1.16%), Dodecanoic acid, 1,1'-biphenyl-4-yl carbonyl methyl ester (1.93%), 9H-Xanthene-9-carboxylic acid phenethyl amide (1.23%), l-Proline, and N-allyloxycarbonyl-, octyl ester (1.05%) were the major compounds identified from the mass spectrum (Fig. 7).

**Identification of fungal isolates**

The culture morphology and spore characteristics of the two fungal isolates were shown in Fig. 8. The MIP-5 isolate had a white cottony mycelium and produced rod shaped conidia with blunt ends, characteristic of *Colletotrichum* spp. Similarly, MIP-3 had a pale green mycelium with powdery mass of conidiospores borne on numerous erect sporangiophores, characteristic of *Penicillium* spp. The ITS sequences were amplified, sequenced and used to identify the fungal isolates. The blast search for the ITS sequence of MIP-3 (575bp) rendered *Penicillium citrinum* as the closest match with >98% sequence identity and 98% query coverage. The isolate MIP-3 resolved along with *Penicillium citrinum* in the maximum likelihood phylogenetic tree with 73% bootstrap support (Fig. 9). Similarly, the blast search for the ITS sequence of MIP-5 (548bp) rendered several *Colletotrichum* spp. as the closest match with 99.45% sequence identity and 100% query coverage.

![Fig. 3.](image-url)

Fig. 3. Shows the data of spectral and chromatographic analysis of putative fungal taxol from *Colletotrichum* sp. MIP-5 and Paclitaxel. (A) UV-Visible spectrum (B) FTIR spectrum and (C & D) HPLC Chromatogram of Paclitaxel and putative fungal taxol from *Colletotrichum* sp. MIP-5.
coverage. The isolate MIP-5 resolved along with C. queenslandicum, C. ti, C. arecicola, and C. tropicale and clade separation between them was not well supported (Fig. 9). Hence, it was identified only at the genus level to avoid erroneous naming. ITS sequences of the two isolates generated in the study was deposited in Genbank for global access under MZ955452 (Penicillium sp. MIP-3) and MZ959110 (Colletotrichum sp. MIP-5).

DISCUSSION

Endophytic fungi are widely recognized as source of novel bioactive secondary metabolites of significant biotechnology and therapeutic values. Intricate invasion and defence strategies of these cryptic organisms render them greater arsenal of metabolites to evade the plant defence, colonize and subsequently confer plant protection against pathogen and pest, in some cases confer physiological benefits like adaptation to heat, drought and salinity stresses.

Taxol, claimed to be the first billion-dollar anticancer drug revolutionized the endophytic fungal research and exploration efforts grew exponentially. Taxol producers' list spans across unrelated taxa and more interestingly were reported from diverse hosts that included both gymnosperms and angiosperms plants of varied ecosystems. Also, several intermediary compounds and taxol derivatives collectively termed as Taxanes were reported from endophytic fungi with potential therapeutic values. Taxanes

**Fig. 4.** (A) DCM extract of Penicillium sp. MIP-3 showing fluorescence. (B) TLC analysis of extracts harvested at different days (5th, 10th 15th & 20th day). (C) partially purified fraction resolved on pre-coated silica plates, (D) authentic riboflavin.

**Fig. 5.** (A) Shows the fluorescence spectral features of the partially purified fluorescent compound from Penicillium sp. MIP-3 (B) Contour graph showing the emission maximum.
are used in palliative treatment of cancer, because of their anticancer activity and unique mechanism of action by stabilizing the microtubules.\textsuperscript{59} In the present study, presence of taxane in the fungal extracts were screened based on TLC plate by co-migrating them along with paclitaxel (Sigma-Aldrich). Among 31 endophytic fungal isolates, only five isolates had bands with similar \( R_f \) value of the paclitaxel and were considered as potential taxane producers. The spectral (UV-Visible and FTIR) and chromatographic (HPLC) data, suggested \textit{Colletotrichum} sp. MIP-5 as a putative taxol producer. Despite the development of genetic screens for taxol, analytical methods were needed to ascertain the taxol production in real time. The primary reason being the temporary inactivation of the gene cassette due to the loss of environmental cues or permanent loss due to gene mutations or disruptions. Furthermore, the complexity of the biosynthetic pathway and lack of complete knowledge about the same impedes the genetic screening methods and analytical methods continue to garner wide support.\textsuperscript{60} Hence in this study, analytical methods were employed for ascertaining the putative taxol producers. Erstwhile, \textit{Colletotrichum} genera has been reported to produce taxol\textsuperscript{55} and also a rich repertoire of other bioactive metabolites like 6-isoprenylindole-3-carboxylic acid, Colletotric acid, Colutellin A, Piperine, Asiaticoside, Quinine, Colletotrichine, Colletopeptides, Mellein, and Brevianamide F.\textsuperscript{61-68} The species boundaries in this cosmopolitan genera have been obscure and although considerable clarification have been reported through multilocus phylogeny,\textsuperscript{69} re-evaluation of the \textit{Colletotrichum} taxonomy would be inevitable to ascertain the species identities.

Several fluorescent and bio-luminance molecules, primarily from mushrooms have been reported. A fluorescent molecule, ergosta-4,6,8
(14),22-tetraen-3-one, steroid derivative, was reported from *Alternaria alternate*.\(^{10,72}\) Territrems were lactone derivatives reported from *Aspergillus terreus*, exhibiting blue fluorescence (\(E_m = 420\)nm).\(^{73}\) The mycotoxins from *Penicillium janthinellum*, Janthitrems belong to the indole di-terpenoids class and have been reported to exhibit blue fluorescence (\(E_m = 385\)nm).\(^{31,71}\) The fluorescence spectral feature (\(E_m = 500-520\)nm) of the compound from our isolate *Penicillium* sp. MIP-3 overlapped with Flavin derivatives. A significant drop in the fluorescence intensity and shift in the \(E_m\) was recorded when the compound from *Penicillium* sp. MIP-3 was dissolved in water (\(E_m = 545\)nm) and methanol (\(E_m = 538\)nm). Riboflavin (water soluble vitamin B2), a flavin derivative exhibiting greenish yellow fluorescence (emission range 500-600nm) was reported from several fungi and *Ashbya gossypii* was employed for its industrial production.\(^{74}\) GC-MS based metabolite profiling pointed out to the presence of Latia luciferin (1-Buten-1-ol, 2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, formate, (1E)-) in the extract of *Penicillium* sp. MIP-3. Latia luciferin had been implicated in the bio-luminescence (\(E_m = 536\)nm) of a fresh water limpet.
*Latia neritoides*.\(^{75}\) Luciferins and their associated enzyme systems had been implicated in the bio-luminescence of fungi and other luminescent organisms.\(^{28,76}\) Recently, 3-hydroxyhispidin, a fungal luciferin has been reported to be involved in the bio-luminescence of fungi.\(^{11}\) Harvey\(^{77}\) had reported the luminescence phenomenon accompanying the oxidation of luciferin without luciferase under oxidizing conditions or with gentle heating in alcohol. Recently, Yu et al.\(^{78}\) reported that the *Latia* luciferin was lacking fluorescence potential based on the theoretical

![Fig. 8. Shows the culture and spore (50x magnification) morphology of the fungal isolates on PDA. (A, B) *Colletotrichum* sp MIP-5 (C, D) *Penicillium* sp. MIP-3.](image)

![Fig. 9. Shows a maximum likelihood tree with bootstrap support values reflected on the nodes.](image)
studies of the spectral features, geometrical and electronic structures. Purification and further characterization of the fluorescent molecule from *Penicillium* sp. MIP-3 can shed more information. Nevertheless, this has been the first report of Latia luciferin from fungal source. In addition, DCM extract of *Penicillium* sp. MIP-3 had several biologically active steroid derivatives. β-Sitosterol, a prominent antioxidant, and anticholesteremic drug was the prominently observed metabolites. Recently, β-Sitosterol had been reported for its immunomodulatory effect against SARS-Cov2. The observance of sterol and fatty acid derivatives as the major compounds in GC-MS spectrum could be attributed to the mid-polar DCM extraction procedure employed. Aspidinol, a phloroglucinol derivative with antibacterial activity was also observed. Bioprospecting these organisms hold greater promise due to their ability to produce wide variety of novel and potential compounds.

**CONCLUSION**

In the present study, about 31 fungal isolates were recovered from the *Mangifera indica* leaves. Among five putative taxol producers identified based on TLC, *Colletotrichum* sp. MIP-5 was reported to be a potential producer of taxanes. Mass production and purification of the taxanes could help in elucidating the structural identity of the taxanes and subsequently could serve as a potential source of anticancer metabolites. Also, *Penicillium* sp. MIP-3 was observed to produce fluorescent molecule with Flavin like fluorescence spectrum. Based on GC-MS analysis, Latia luciferin was reported for the first time from this fungal source. In addition, therapeutically active steroid, quinones and phloroglucinol derivatives were also reported from *Penicillium* sp. MIP-3. These molecules could potentially be used in biological applications and needs further studies.

**SUPPLEMENTARY INFORMATION**

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.15.4.43

Additional file: Additional Table S1.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHORS’ CONTRIBUTION**

MP designed and guided the study. EGJ, PS conducted the experiments, analyzed the data and drafted the manuscript. SSK helped with phylogenetic analysis and manuscript preparation. MP reviewed the manuscript.

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**DATA AVAILABILITY**

The sequences generated in the study were submitted in Genbank (M2955452 and MZ959110, awaiting release) and were being processed for global release. All reference sequences employed in the study were retrieved from Genbank and their accession numbers were reflected in the phylogenetic tree and presented in supplementary file, S1.

**ETHICS STATEMENT**

Not applicable.

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