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New record of novel endophyte *Nigrograna hydei* from the Northern Himalayas, India

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The Northern Himalayas are an exceptionally unique and diverse mountain range with respect to flora, fauna and funga. However, most of the region has been poorly explored. During a mycological survey, a rare endophyte was isolated from healthy leaves of *Elaeagnus angustifolia*. The species was identified as *Nigrograna hydei* based on cultural, micro-morphological, and molecular characters. The specimen was recovered as a sterile mycelium and despite trying various sporulation-inducing methods, the isolate failed to sporulate. Therefore, molecular characterisation based on the sequences of both the ITS and LSU region of nuclear ribosomal DNA followed by phylogenetic analysis confirmed the identity of the taxon. So far, this species has only been reported from Thailand and China. This finding adds another record to the world distribution of the species and is the first report from the Indian subcontinent. It also expands our knowledge on its ecology through its association as an endophyte with a new host, *Elaeagnus*, inhabiting a cold desert in Ladakh.

Key words: sterile mycelium, rare, taxonomy, cold desert, Kargil.

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Severní Himálaj je jedinečné horstvo s rozmanitou flórou, faunou i fungou, která ovšem byla a stále je nedostatečně prozkoumaná. Během mykologického průzkumu zde byl ze zdravých listů *Elaeagnus angustifolia* izolován vzácný endofyt, na základě charakteristiky kultur, mikromorfologických znaků a molekulárních dat určený jako *Nigrograna hydei*. Navzdory různým pokusům vyvolat sporulaci zůstal izolát v podobě sterilního mycelia a bezpečné potvrzení jeho identity poskytly až sekvence ITS a LSU úseku nrDNA s následnou fylogenetickou analýzou. Záznamy o výskytu tohoto druhu dosud pocházejí jen z Thajska a Číny, a tak nový nález přispívá k rozšíření jeho známého areálu ve světě a představuje první záznam z indického subkontinentu. Rozšiřuje i poznání ekologie druhu, rostoucího zde endofyticky v hlošině coby novém hostiteli, a to v prostředí chladné pouště v Ladakhu.

Introduction

The genus *Nigrograna* was initially described by de Gruyter et al. (2012), including a single species, *N. mackinnonii* (Borelli) Gruyter, Verkley et Crous, well-recognised as a human pathogen and the causative agent of eumycetoma in Latin America (Ahmed et al. 2018). However, Ahmed et al. (2014) reclassified the genus *Nigrograna* as *Biatriospora* due to its sequence similarity to the type species *B. marina* K.D. Hyde et Borse. Later, Jaklitsch and Voglmayr (2016) reported three new species exhibiting more phylogenetic relationship with *N. mackinnonii* and also forming different ascospores than *Biatriospora marina*. Based on this, a new family for the genus *Nigrograna*, i.e. *Nigrogranaceae* was therefore established. After this, three newly discovered species were added, along with the relocation of one species into this family and *N. mackinnonii* as the type species (previously allocated in the family *Biatriosporaceae*) (Zhang et al. 2020).

Following, four more species earlier described under the genus *Biatriospora* were recombined in the genus *Nigrograna* due to their distinction in phylogeny from *Biatriospora* but close resemblance to *Nigrograna*. These four species were *Biatriospora antibiotica* (M. Kolařík et Kubátová) M. Kolařík, *B. yasuniana* (M. Kolařík et D. Spakowicz) M. Kolařík, *B. carollii* (M. Kolařík et R. Gazis) M. Kolařík, and *B. peruviensis* (M. Kolařík et R. Gazis) M. Kolařík (Kolařík et al. 2017, Kolařík 2018). At present, 23 species of *Nigrograna* are reported according to Index Fungorum (http://www.indexfungorum.org/Names/Names.asp).

Endophytes are micro-organisms living inside many plant species and spending at least a part of their life cycle without causing any symptoms of infection (Hardoim et al. 2015). It is estimated that approximately 3 million species of endophyte currently exist while only 150,000 fungal species have been described to date (Bhunjun et al. 2023). The plant species *Elaeagnus angustifolia* L. is distributed in the high-altitude Himalayan regions of India, such as Ladakh and Uttarakhand. This plant is native to Eurasia and commonly called Russian olive or oleaster and vernacularly called sersing in Ladakh and Giwain in Uttarakhand. This is a small tree having brown coloured branches with young silvery shoots and green alternately arranged leaves and edible fruits (Gairola et Biswas 2008, Raj et al. 2010, Enescu 2018). It is an ethnomedicinally important plant with many pharmaceutical properties and is well known for its survival at high altitudes and extreme habitats. Decoctions of the various plant parts, such as flowers, fruits, leaves and bark have been used by local people to treat different illnesses (Hamidpour et al. 2017).

This plant has so far not been explored for its endophytic diversity worldwide. In an attempt to investigate its endophytic microbiome, a range of endophytic isolates (mostly sterile) have been recovered. Further, the taxonomic identity of such sterile mycelia has been confirmed with the aid of cultural and morphological

features and molecular methods. In the present study, we report *Nigrograna hydei* J.F. Zhang, J.K. Liu et Z.Y. Liu as a new record from India with the aim to unravel the endophytic diversity of this pharmaceutically important plant inhabiting an extreme habitat. In various studies, based on ecology and evolutionary relationship between host and endophyte, it has been well demonstrated that fungal endophytes play a crucial role in the survival of their host plant in extreme habitats and exhibit beneficial roles in their survival (Sung et al. 2008, Doilom et al. 2017).

MATERIAL AND METHODS

Selection of the plant. The host plant for isolation of endophytes was selected from the village of Wakha (34°22'08" N and 76°24'31" E; 3,497 m a.s.l.) 40 km southeast of Kargil in the Union Territory of Ladakh (34°33'14" N and 76°08'06" E), a region usually acknowledged as an extremely cold and arid region. Samples of the selected plant were collected aseptically by using a random sampling method and were packed in sterile polythene bags for efficient transportation to the laboratory.

Isolation of fungal endophytes. The isolation was carried out within 24–48 hours after sample collection. The leaves were surface-sterilised following the sterilisation procedure given by Petrini (1986) with slight modifications. The leaves were rinsed thoroughly under running tap water and immersed in 70% ethanol for 3 minutes, followed by washing with sodium hypochlorite (2%) for 2 minutes and a second washing with 70% ethanol for 1 minute. Finally, these surface-sterilised leaves were rinsed twice with sterile distilled water. Thereafter, complete drying of the leaves was performed under folds of filter paper in a laminar chamber.

Afterwards, each leaf was cut into (2 × 2 mm) segments using a sterile blade or scalpel and placed on potato dextrose agar (PDA) plates supplemented with streptomycin (250 mg/l). The inoculated leaf segments were further incubated at 28 ± 2 °C until growth of mycelium from the cut ends of the leaf segments (Fig. 2a). The leaf segments were then transferred to fresh PDA plates for purification and identification.

Sporulation induction and morphological examination. The morphological examination included visible morphological characters, such as colony colour, size, texture, growth pattern, and presence or absence of exudates. A compound light microscope (Magnus, India and Leica DM2000 LED, Germany) and an inverted compound microscope (Evos® FL Cell Imaging System, Thermo Fisher Scientific, Waltham, USA) were used for morphological studies. Morphological examination of the fungal mycelium was carried out using a 1000× magnification lens. Despite using different nutrient media (source: Himedia, Mumbai, Maharashtra, India), i.e. potato dextrose agar (PDA), oat meal agar (OMA), water agar (WA), malt extract agar (MEA), corn meal agar (CMA), and the use of alternating light and dark cycles for inducing sporulation, the isolate failed to sporulate.

The culture, maintained as ELS1-2022, is deposited in the Plant Pathology Lab, Department of Botany, University of Jammu, and in National Centre for Microbial Resource, Pune, India.

DNA extraction, amplification, sequencing and phylogenetic analysis. Genomic DNA was extracted from mycelia by using the phenol/chloroform extraction method (Sambrook et al. 1989). The extraction was followed by polymerase chain reaction (PCR) amplification of the nrITS regions by using universal primers (both forward and reverse primers) ITS1 [5'-TCC GTA GGT GAA CCT GCG G-3'] and ITS4 [5'-TCC TCC GCT TAT TGA TAT GC-3'] (White et al. 1990) and the nrLSU region by using LR0R [5'-GGT CCG TGT TTC AAG AC-3'] and LR5 [5'-ATC CTG AGG

GAA ACT TC-3'] (Vilgalys et Hester 1990). The amplified PCR product was then purified by using PEG-NaCl precipitation and followed by direct sequencing on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the manufacturer's instructions. To read both ends carefully, sequencing was carried out through both the ends. The Lasergene package was used to perform the assembly of sequences followed by editing and NCBI (National Centre for Biotechnology Information) blast (Burland 2000, Boratyn et al. 2013).

A consensus sequence was made and edited in the BioEdit software version 7.2 (Hall 1999). The sequence was blasted in NCBI database using the site www.ncbi.nlm.nih.gov/BLAST in order to get the closest sequences of the genes for preparing the dataset for construction of the phylogenetic tree. Alignment of the genes was performed using MAFFT version 7 (https://mafft.cbrc.jp/alignment/server/; Katoh et Standley 2013). The alignment editing was performed with the BioEdit software. The final dataset of sequences was made and used to construct phylogenetic relationships between different sequences. The tree was constructed by means of the maximum likelihood method and Kimura 2 parameter model (Kimura 1980) using the MEGA 11 software including 1000 replication bootstrap values (Tamura et al. 2021). The dataset consisted of 19 strains of *Nigrograna* using *Occultibambusa pustula* (MFLUCC 11-0502) as outgroup, see Tab. 1.

Tab. 1. Details of fungal isolates used for phylogenetic analysis along with their GenBank accession numbers for nrITS and nrLSU sequence data.

Taxon	Strain	GenBank accession number		Country	Reference
		ITS	LSU		
Nigrograna antibiotica	CCF 4378	NR_158296	NG_058663	Czech Republic	Kolařík et al. 2017
Nigrograna aquatica	MFLUCC 17-2318	NR_171881	NG_073796	Thailand	Wei et al. 2020
Nigrograna cangshanensis	MFLUCC 15-0253	NR_155486	NG_059778	China	Tibpromma et al. 2017
Nigrograna fuscidula	MF7	KX650550	KX650550	Austria	Jaklitsch et Voglmayr 2016
Nigrograna fuscidula	MF3	KX650549	KX650549	Austria	Jaklitsch et Voglmayr 2016
Nigrograna fuscidula	MF9	KX650552	KX650552	Italy	Jaklitsch et Voglmayr 2016
Nigrograna hydei	MFLU 18-2073	NR_172415	MN387227	Thailand	Zhang et al. 2020
Nigrograna hydei	**ELS1-2022**	**OP741100**	**OP741109**	**India**	**This study**
Nigrograna impatientis	MFLU 18-2072	NR_172416	MN387228	Thailand	Zhang et al. 2020
Nigrograna mackinnonii	CBS 674.75	NR_132037	GQ387613	Venezuela	Ahmed et al. 2014
Nigrograna mackinnonii	E5202H	JX264157	JX264157	Ecuador	Shaw et al. 2015
Nigrograna mycophila	TDK	KX650555	KX650555	Denmark	Jaklitsch et Voglmayr 2016
Nigrograna mycophila	MF6	KX650554	KX650554	Denmark	Jaklitsch et Voglmayr 2016
Nigrograna mycophila	MF5	KX650553	KX650553	Austria	Jaklitsch et Voglmayr 2016
Nigrograna norvegica	TR8	KX650556	KX650556	Norway	Jaklitsch et Voglmayr 2016
Nigrograna obliqua	BW4	KX650557	KX650557	Austria	Jaklitsch et Voglmayr 2016
Nigrograna obliqua	MRP	KX650561	KX650561	Austria	Jaklitsch et Voglmayr 2016
Nigrograna obliqua	KE	KX650558	KX650558	Austria	Jaklitsch et Voglmayr 2016
Nigrograna thymi	MFLU 17-0497	NR_160462	NG_064431	Italy	Hyde et al. 2017
Occultibambusa pustula	MFLUCC 11-0502	KU940126	KU863115	Thailand	Dai et al. 2017

RESULTS

Out of 155 surface-sterilised leaf segments, 61 endophytic isolates were recovered with an overall colonisation frequency of 39.35%. Out of these, *Nigrograna hydei* (ELS1-2022) was isolated only once, exhibiting a low colonisation frequency of 0.64%.

Phylogenetic analysis

Using Megablast search in the NCBI database, the closest matches corresponding to the nrITS sequence of ELS1-2022 included *N. hydei* DUCC 15293 (99.81%) and *N. mackinnonii* NTOU 4480 (99.81%). However, the maximum similarity percentage (i.e. 100%) was exhibited by the *N. hydei* (MFLU 18-2073) type material. The closest hits in GenBank using the nrLSU sequence were *N. mackinnonii*

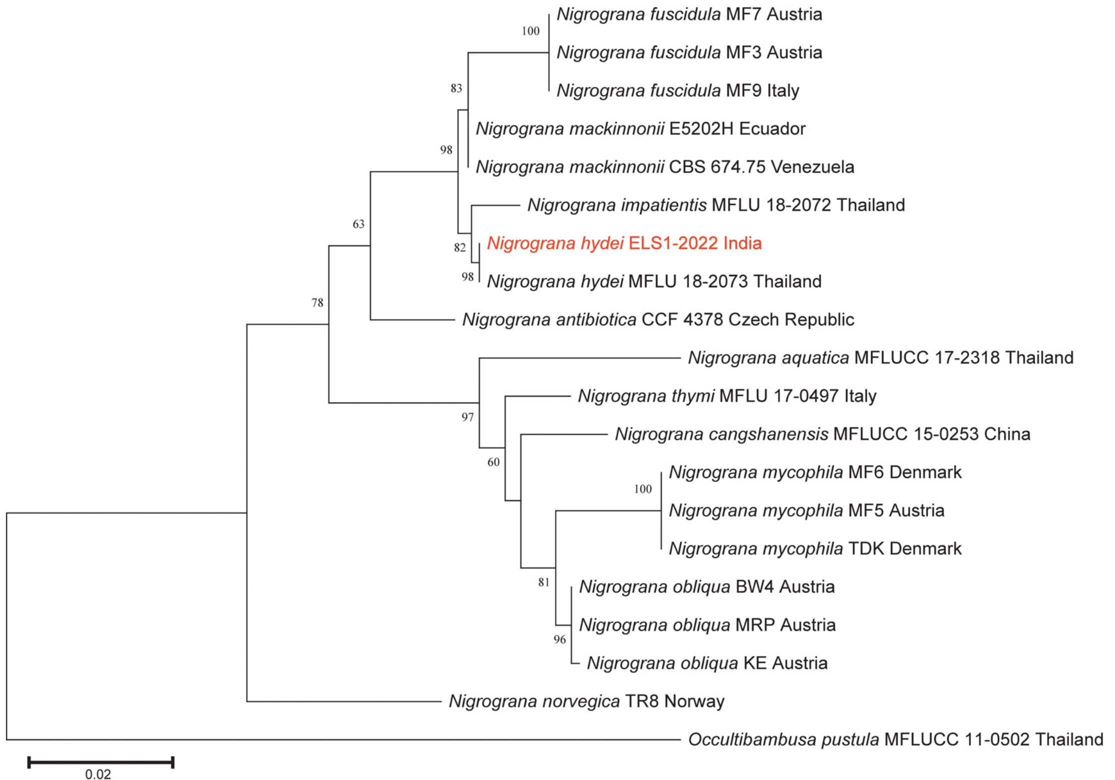

Fig. 1. Phylogram inferred from maximum likelihood (ML) analysis based on combined sequences of the nrITS and nrLSU dataset. Branches with >50% values are shown both below and above the nodes. Scale bar = 0.02.

UTHSC:DI16-241, *N. hydei* DUCC 15293 and *N. chromolaenae* MFLUCC 17-1437 (each 99.81%), while *N. impatientis* MFLU 18-2072 exhibited 99.64% similarity. After complete phylogenetic analysis of combined nrITS and nrLSU of the current isolate, ELS1-2022 grouped strongly with *N. hydei* MFLU 18-2073 (GenBank nrITS NR_172415 and nrLSU MN387227) with 98% bootstrap support (Fig. 1).

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Cultural characteristics. Colonies of ELS1-2022 on PDA floccose, dome-shaped, slightly heaped at centre, whitish grey with brownish shade, aerial mycelia reaching up to 30 mm in diameter after incubation at 28 ± 2 °C for 15–20 days; reverse dark brown at centre, periphery turning yellowish. On MEA, dome-shaped, slightly heaped at centre, steel grey, aerial mycelia reaching 15 mm diameter after 15 days at 28 ± 2 °C; reverse with greenish centre and periphery turning white. Colonies on OMA floccose, dome-shaped, slightly heaped at centre, steel grey, aerial mycelia attaining up to 28 mm in diameter after 15–20 days at 28 ± 2 °C; reverse greenish black, with off-white periphery. Colonies on WA were restricted in growth, floccose, greenish black, reaching up to 25 mm in size after 15–20 days of incubation at 28 ± 2 °C, reverse light grey (Fig. 2).

Morphological characteristics. In the present isolate ELS1-2022, the hyphae were golden brown or olivaceous brown, septate with 3–4 µm wide branches, and intercalary chlamydospores present.

Habitat and distribution. Endophytic in leaves of *Elaeagnus angustifolia* growing in the cold arid region of Kargil, Ladakh (India).

DISCUSSION

Nigrograna (*Ascomycota*, *Pleosporales*) is the only genus in the family *Nigrogranaceae* with ecologically diverse species growing in a variety of environments, such as terrestrial, estuarine and marine habitats (Kolařík 2018). For example, *N. aquatica* (on submerged wooden log), *N. cangshanensis* (on decaying wood), *N. chromolaenae* (on *Chromolaena odorata*), *N. rhizophorae* (on *Rhizophora* sp.), *N. samueliana* (on decaying mangrove wood), and *N. thymi* (on *Thymus oenipontanus*) have been reported as saprophytes (Hyde et al. 2017, Tibpromma et al. 2017, Dayarathne et al. 2020, Dong et al. 2020, Mapook et al. 2020). On the other hand, *N. antibiotica*, *N. carollii*, *N. mackinnonii*, *N. peruviensis* and *N. yasuniana* have been reported to exist as endophytes within hosts growing in tropical and subtropical regions, such as *Ulmus laevis*, *Hevea brasiliensis*, *Guazuma ulmifolia*, *Virola* sp. and *Conceveiba guianensis*, respectively (Shaw

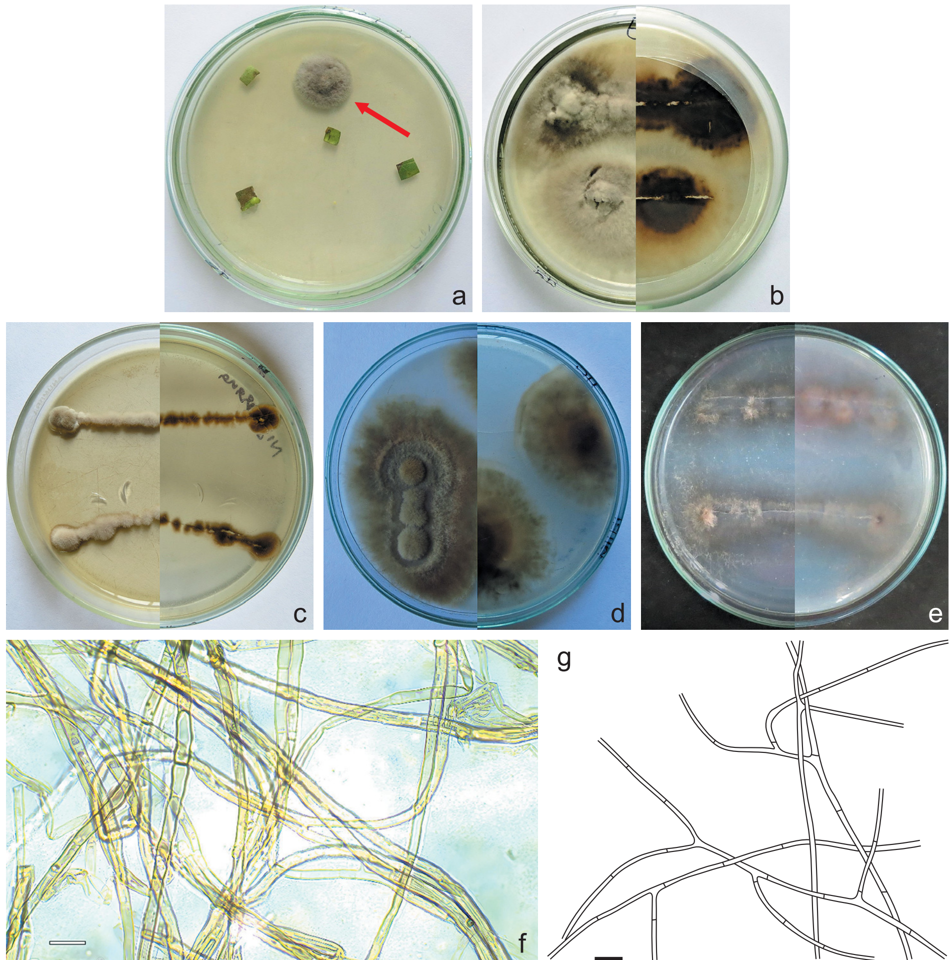

Fig. 2. Cultural characteristics of *Nigrograna hydei* (ELS1-2022): **a** – isolation plate showing emergence of mycelium (arrow) from a surface-sterilised leaf segment; **b** – colonies on PDA (front left; reverse right); **c–e** – colonies on MEA, OMA, WA, respectively (front left; reverse right); **f** – sterile mycelium under microscope; **g** – sterile mycelium, camera-lucida diagram. Bars (f, g) = 10 µm. Photos and drawing by Shivani Digra.

et al. 2015, Kolařík 2018). Endophytic isolate ELS1-2022 was isolated from a plant growing in a cold arid region indicating its cold-tolerating ability and thereby its habitat diversity. Some researchers have reported the pathogenic potential of some species, such as *N. fuscidula*, *N. magnoliae*, and *N. mackinnonii* as pathogens on *Sambucus nigra*, *Magnolia denudata* and humans, respectively (Jaklitsch et Voglmayr 2016, Ahmed et al. 2018, Wanasinghe et al. 2020). Interestingly, some

species of *Nigrograna*, such as *N. norvegica* and *N. obliqua*, have also been reported to be mycophilic (Jaklitsch et Voglmayr 2016).

Different species of *Nigrograna* have been recorded from various regions of the world, such as Asia, Australia, Europe, North, Central and South America (Kolařík 2018). The species *N. hydei* was first reported from Thailand as a saprophyte from an unidentified host (Zhang et al. 2020). The colony of that isolate was grey and brown to dark pigmented in the reverse but whitish grey with a brownish shade and dark brown in the reverse with a yellow periphery in the present isolate. It also showed sporulation on PDA medium. However, its asexual morph was not observed, whereas ELS1-2022 did not show any form of sporulation, whether asexual or sexual. Isolate ELS1-2022 showed some resemblances with the above-mentioned species from Thailand in terms of hypha width (1.6–2.5 µm), the aerial mycelium with entire edges, and olivaceous or golden brown hyphae. However, the asexual morph of the species is still unknown.

Nigrograna hydei has also been enlisted as one of the airborne fungi out of 1015 fungal strains isolated from different outdoor environments in a recent report from China, but a detailed description of the species is lacking, only mentioned as a genus in a Venn diagram and a phylogram. The information about the species was confirmed upon searching the given accession number (ON712590) in GenBank (Nageen et al. 2023). This further indicates a broad habitat range of the genus *Nigrograna*.

ELS1-2022 was observed to be a slow-growing endophyte not responding to alternating light and dark cycles or to diverse media (PDA, MEA, OMA, and WA) to induce sporulation. Isolation of sterile mycelia has been a very common practice during studies of fungal endophytes. Identification of any species of *Nigrograna* based on a morphological approach is definitely unsatisfactory, which makes its molecular characterisation a crucial step forward to identification at the species level (Jaklitsch et Voglmayr 2016). Therefore, phylogenetic analysis based on nrITS and nrLSU was used to confirm the identity.

The survival of plants in habitats with extreme environmental conditions has led to extensive research in this field. As a result, the role of fungal symbiosis in survival of such plants in these stressed habitats via a range of mechanisms has been confirmed. Such environments are also expected to harbour a plethora of unique microbes including fungi, as observed in the present study. Therefore, similar explorations should be carried out to discover the rare and diverse microbiomes associated with plants which can act as a reservoir of bioactive secondary metabolites with various applications in the fields of medicine, agronomy, cosmetics and nutrition (Suryanarayanan et al. 2012).

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