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EXPLORING ORCHID MYCORRHIZA: ISOLATION AND IDENTIFICATION FROM AN EPIPHYTIC ORCHID, *LUISIA TRICHORHIZA* (HOOK.) BLUME

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Abstract

Endophytic fungi are integral components of orchid biology, contributing to nutrient acquisition, stress tolerance, and ecological adaptations. In the present study, root-associated fungal endophytes were isolated and characterized from an epiphytic orchid, *Luisia trichorhiza* (Hook.) Blume, collected from the Chamoli district of Uttarakhand, India. Fungal isolates were cultured on Potato Dextrose Agar (PDA) medium and examined using morphological, anatomical, and molecular approaches. Distinct colony morphologies and micromorphological traits indicated the presence of two taxonomically different fungal isolates. Molecular identification based on sequencing of the internal transcribed spacer (ITS) region revealed that isolate LT-1 showed 99.58% sequence similarity with *Neocosmospora rubicola*, whereas isolate LT-2 exhibited 94.76% similarity with *Thelonectria blackeriella* and therefore, the latter was conservatively identified up to the genus level as *Thelonectria* sp. The corresponding ITS sequences were deposited in NCBI GenBank under accession numbers PX640217 (LT-1) and PX735845 (LT-2). Phylogenetic analyses using the Maximum Likelihood method further supported the taxonomic placement of both these isolates within their respective lineages. Anatomical investigations confirmed that fungal colonisation was restricted to the root system, with the formation of characteristic intracellular pelotons within cortical cells, particularly in the root hair zone, while aerial tissues remained uninfected. The coexistence of both common and relatively rare fungal taxa highlighted the complexity of the root-associated mycobiome of *L. trichorhiza*. The present findings provide novel insights into orchid-fungal associations and establish a baseline for future studies on orchid conservation, *in vitro* symbiotic germination, and biotechnological potential of endophytic fungi.

Introduction

ENDOPHYTIC FUNGI are microorganisms that inhabit the internal tissues of living plants without inducing noticeable injury or pathogenic infection in their hosts (Fouda *et al*., 2015). These organisms are diverse and omnipresent in nature, residing in many tissues, including the host plant's leaves, stems, roots, flowers, and fruits. Fungal endophytes are also recognized as prolific makers of several bioactive compounds (Strobel and Daisy, 2003). Fungal endophytes have attracted interest due to the discovery of numerous endophytic metabolites possessing medicinal characteristics, including antibacterial, antifungal, and antioxidant chemicals (Pawle, 2014). Consequently, fungal endophytes are increasingly recognized as a significant source of novel natural substances and their potential applications in medicine, agriculture, and industry. The endophyte-orchid relationship was first established for the survival of these plants as the absence of endosperm in their seeds necessitates their reliance on these endosymbionts for their germination and growth, in nature (Chua *et al*., 2022). Recently, there has been an exploration of chemicals, particularly due to the growing recognition of the medical properties of the orchid plants. It has been hypothesized that endophytes residing in host plants may synthesize therapeutical chemicals analogous to their host plants (Xing *et al*., 2011).

The family Orchidaceae is a broad and diverse family of angiosperms, primarily recognized for their distinctive flower morphologies. Orchids are a significant source of bioactive substances utilized in several traditional medicine to treat myriad diseases, ailments, and impairments (Kumari and Pathak, 2025a,b; Pant, 2013; Sharma and Pathak, 2024). Endophytism involving microorganisms is a complex phenomenon associated with orchids, facilitating germination and tolerance to harsh climatic conditions. Such fungi linked with orchids primarily belong to orchid mycorrhiza. The diversity of endophytic fungi from Ascomycota and Basidiomycota has also been abundant (Varma *et al*., 2017). Orchids mainly rely on a fungal companion for their carbon source during the early stages of life; however, at their mature stage, this relationship may aid in coping with adverse environmental conditions such as drought, salt, and oxidative stress (Pant *et al*., 2019; Varma *et al*., 2017).

Mutualistic ecological mechanisms, including pollination and fungal symbiosis, are recognized as the catalysts of orchid diversification (Givnish *et al*., 2016; Waterman and Bidartondo, 2008). It is hypothesised that fungi have co-evolved with several orchids, addressing their requirements through specialized ecological interactions (Givnish *et al*., 2016; Leake and Cameron, 2012). Endosperm-less seeds are a common characteristic in the family Orchidaceae, and these are disseminated with immature embryos, typically at the

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globular stage (Eriksson and Kainulainen, 2011). The embryo's nutritional needs, first provided by the endosperm, are ultimately fulfilled by specialized mycorrhizal fungi, referred to as Orchid Mycorrhizae (Rasmussen and Rasmussen, 2009). Notably, the majority of epiphytic orchids favour non-mycorrhizal endophytes that inhabit the substrate present on tree trunks (Liu *et al*., 2010).

Literature studies indicate that non-mycorrhizal endophytes though promote mycorrhizal colonisation during initial developmental phases (Yuan *et al*., 2009), function as pathogens in the subsequent stages of seedling growth (Delaye *et al*., 2013). In the mature phase, fungi assimilate sugars and carbon reserves for mycelial development while simultaneously supplying growth-promoting chemicals, thus guaranteeing mutual benefits for both interacting partners (Cameron *et al*., 2006). Endophytic fungi synthesise diverse bioactive chemicals near orchid roots, providing resistance against infections (Vaz *et al*., 2009).

Hence, it is essential to investigate these interactions and examine the correlations across the life cycle of an orchid. Verifying the data through the examination of orchid communities in their native settings will be essential for comprehending the survival and distribution pattern of orchids. Non-mycorrhizal endophytic fungi serve a specific function during the advanced phases of orchid development and are essential at various periods during the life cycle of an orchid species (Meng *et al*., 2019). Although orchid-mycorrhiza symbiosis often has a coevolutionary foundation (Martos *et al*., 2012), this has not been investigated for orchid-non-mycorrhizal endophyte symbiosis.

The present study examined the identification and characterisation of orchid-endophytic fungal associations in an epiphytic species, *Luisia trichorhiza* (Hook.) Blume. The native range of this species is the Indian Subcontinent extending to Thailand and it grows primarily in the subtropical biome (POWO, 2025). Although, *L. trichorhiza* has not yet been evaluated (NE) under the IUCN Red List, several species of *Luisia* face local declines in their natural populations due to habitat disturbance, forest degradation, and loss of epiphytic substrates (Givnish *et al*., 2015; Phelps *et al*., 2012; Swarts and Dixon, 2009). Despite its ecological sensitivity, almost no information is available on the identity, diversity, or functional significance of its root-associated endophytes. Understanding the fungal endophytes of *L. trichorhiza* is important because i), No comprehensive reports exist on its root-associated endophytic community, and most studies on orchid endophytes have focused on *Bletilla*, *Cymbidium*, *Dendrobium*, and *Vanilla*, leaving *Luisia* largely unexplored as also earlier indicated by a few authors (Herrera *et al*., 2010; Xing *et al*., 2011; Yuan *et al*., 2009); ii), Epiphytic orchids frequently harbour rare or specialized fungal endophytes that enhance adaptation to nutrient-poor canopy habitats (Givnish *et al*., 2016; Liu *et al*., 2010); iii), Endophytic fungi play essential role in symbiotic seed germination and orchid reintroduction programmes, making them crucial for the conservation of RET (rare, endangered, and threatened) species (Rasmussen and Rasmussen, 2009; Zettler *et al*., 2017) and; iv), Uncommon or lesser-known endophytes may possess unique biochemical and biotechnological properties, including antimicrobial and antioxidant activities as also mentioned by a few authors (Chua *et al*., 2022; Pawle, 2014; Strobel and Daisy, 2003).

The present study aimed to investigate orchid mycorrhiza, with the specific objective of isolating and identifying the mycorrhizal fungi associated with the roots of the epiphytic orchid *L. trichorhiza*. This was achieved using a combination of anatomical, morphological, and molecular techniques. The present investigation provides the first insights into the species endophytic diversity and establishes a baseline for future efforts in orchid conservation, *in vitro* symbiotic germination, and bioprospecting.

Material and Methods

Sample Collection

Fresh mycorrhizal roots of *L. trichorhiza* were collected in airtight plastic bags from naturally occurring plants from Mandal, Chamoli Disrict, Uttarakhand, India (30°302 N latitude and 79°402 E longitude) (Fig. 1) in month of September-October and utilized for the isolation of fungal endophytes within 24 hr of collection. The root samples were cleaned under running tap water to eliminate dust particles and debris from the root surface, then cut into thin transverse slices at various regions of the root and examined under a microscope after staining with lactophenol cotton blue to confirm the existence of fungal pelotons. The roots exhibited the presence of fungal pelotons, which were utilized for the isolation of fungal endophytes.

Anatomical Studies

Anatomical investigations of *L. trichorhiza* revealed that the fungal colonisation was confined exclusively to the root system, while aerial organs such as stems and leaves remained entirely free of infection. Staining with lactophenol cotton blue provided unambiguous visualisation of hyphal penetration so as to highlight

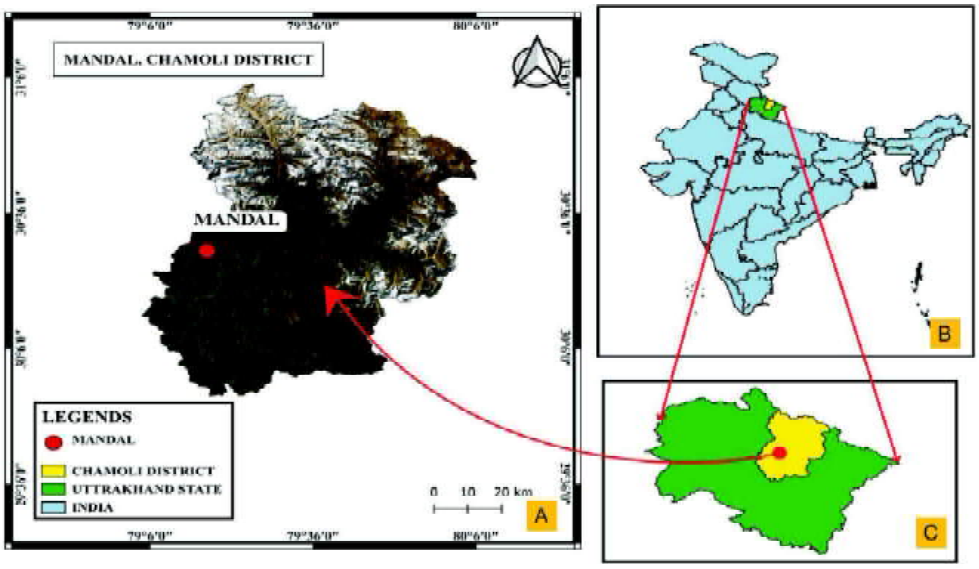

Fig. 1A-C. Location map of study area: A, Satellite view of Mandal region in Chamoli district (Uttarakhand, India) showing the sampling site; B, Location of the Uttarakhand state; C, Map of Uttarakhand showing Chamoli District and the study site (Mandal) indicated as red.

the progressive development of compact intracellular pelotons, which are considered the hallmark of orchid-fungal symbiosis. A comparative assessment of developmental stages including the peloton formation was made for the younger and mature roots.

Isolation of Fungal Endophytes

Root samples were obtained from presently investigated orchid species. Segments measuring up to 5 cm were removed from the apical region, as the endophytic variety was at its zenith in that area followed by Zettler *et al*. (2017). Samples underwent surface sterilization in the laboratory utilising a standard procedure (adapted from Otero *et al*., 2004) consisting of; (i) 5 mins wash under running tap water, (ii) 3 mins rinse with 4% sodium hypochlorite, and (iii) 2 mins rinse with 70% ethanol. The samples were removed from the velamen and outer layers using a sterile surgical blade. Thereafter, 1 cm segments were excised from the cortical area. The samples were subsequently inoculated into 90 mm petri plates containing Potato Dextrose Agar medium (HiMedia Laboratories Pvt. Ltd., Mumbai). Hyphae originating from the root segments were isolated and sub-cultured on fresh PDA nutrient media plates to cultivate pure colonies of each species. The cultures were maintained at 25±2°C for two wks, subjected to 12 hrs alternating cycles of light and darkness.

Isolation of DNA

Fungal mycelia were collected from 14-day-old cultures grown on PDA. Genomic DNA was extracted from the corresponding mycelia utilising the HiPurATM Fungal DNA Purification Kit (HiMedia Laboratories Pvt. Ltd., Mumbai) in accordance with the kit's instructions. The internal transcribed spacer (ITS) region, a universal marker, was selected for the molecular characterisation of fungal taxa (Schoch *et al*., 2012). The primer sequences for the ITS1-ITS4 region were derived from White *et al*., 1990. The target region was amplified via polymerase chain reaction (PCR) in a MyCyclerTM thermal cycler (Bio-Rad Laboratories, USA). The 25 mL reaction comprised: 12.5 mL of 2x GoTaq Green Master Mix (Promega Corporation, USA), 1 mL each of forward primer, reverse primer, DNA template, and 9.5 mL of nuclease-free water. The PCR cycle for DNA amplification was configured as follows: Commence with initial denaturation at 95°C for 3 mins, followed by 30 cycles including denaturation at 94°C for 1 min, annealing at 50°C for 30 sec, and extension at 72 °C for 30 sec. The procedure was succeeded by a final extension at 72°C for 7 mins, followed by storage at 4°C. The amplified PCR products were subsequently analysed using a 1.5% agarose gel. The bands were observed using a UV transilluminator. Subsequent to the validation of PCR fragments, the bands were eluted and purified using a DNA elution kit (Qiagen, Germany).

Results and Discussion

In the present study, root-associated fungal endophytes were isolated and characterized from an epiphytic orchid, *L. trichorhiza* which was collected from the Chamoli district of Uttarakhand, India. Fungal isolates were cultured on PDA nutrient medium and examined using morphological, anatomical, and molecular approaches.

Anatomical Observations

The detailed transverse sections of root demonstrated the presence of characteristic fungal pelotons distributed within cortical cells (Fig. 2B-C), confirming their role as diagnostic markers of orchid mycorrhizal interactions. The most intense colonisation was observed in the root hair zone, where hyphal structures were clearly visible entering root hair (Fig. 2D) and subsequently breaching the epidermal layer (Fig. 2G-H) so as to advance into underlying tissues. Staining

with lactophenol cotton blue provided unambiguous visualisation of hyphal penetration (Fig. 2E-F) and these observations highlighted the progressive development of compact intracellular pelotons, which are considered the hallmark of orchid–fungal symbiosis. Once established inside cortical tissues, the hyphae formed organised pelotons (Fig. 2I) that likely facilitate nutrient exchange between host and endophyte. By contrast, the apical meristematic tip remained completely devoid of infection, suggesting thereby spatial restriction of colonisation within root tissues. A comparative assessment of developmental stages indicated that younger roots displayed substantially higher levels of fungal entry and peloton formation as compared to the mature roots, suggesting thereby that colonisation is initiated more actively during early root ontogeny. These observations collectively emphasise the anatomical specificity and developmental regulation of fungal colonisation in *L. trichorhiza*, underscoring its ecological importance and functional role in sustaining orchid endophyte symbiosis.

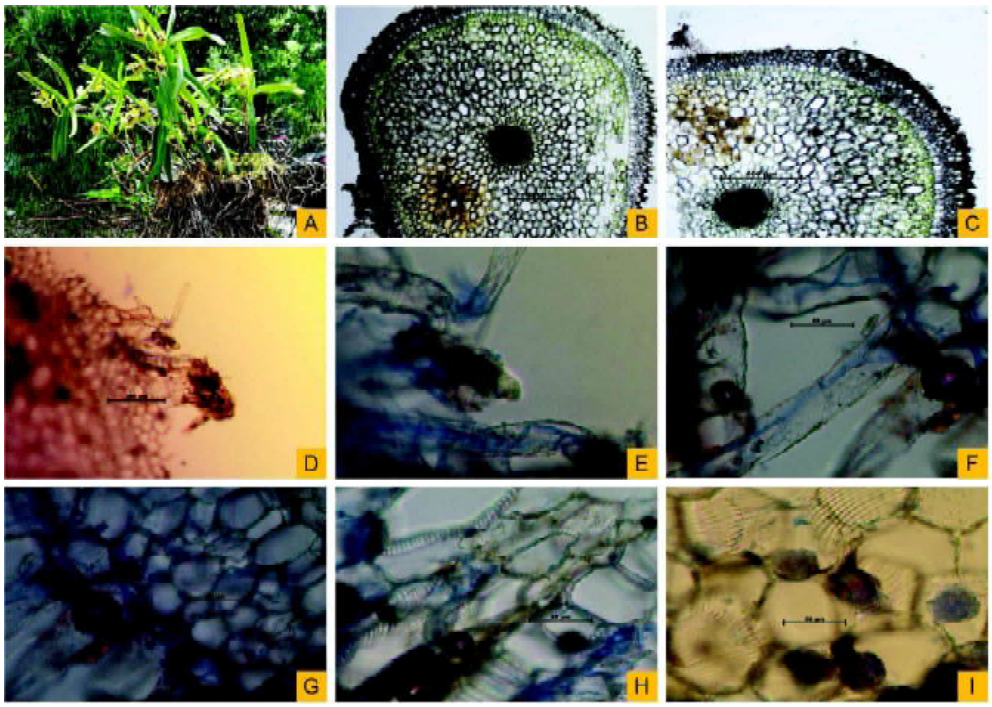

Fig. 2A-I. Orchid Mycorrhiza in *Luisia trichorhiza*: A, Plants in bloom with aerial roots; B-C, A part of Transverse sections of root showing fungal peletons within the cortical region (4x); D, Root hair colonized by fungal hyphae (10x); E-F, Entry of fungal hyphae into root hair (stained with Lactophenol Cotton Blue) (40x); G-H, Penetration of fungal hyphae from root hair into the epidermal cells (40x); I, Formation of intracellular pelotons within the cortical cells (40x).

Morphological (Classical) Observations

Two morphologically distinct fungal endophytes were isolated from the roots of *L. trichorhiza* and cultured on PDA. The isolates [*Neocosmospora rubicola* (25B112_013 LT-1) and *Thelonectria blackeriella* (25B112_014 *LT-2*)] exhibited marked divergence in both macroscopic and microscopic attributes. Isolate 25B112_013 (LT-1) formed a white, floccose colony with a cottony surface texture; the reverse colony displayed uniform brown pigmentation without evident

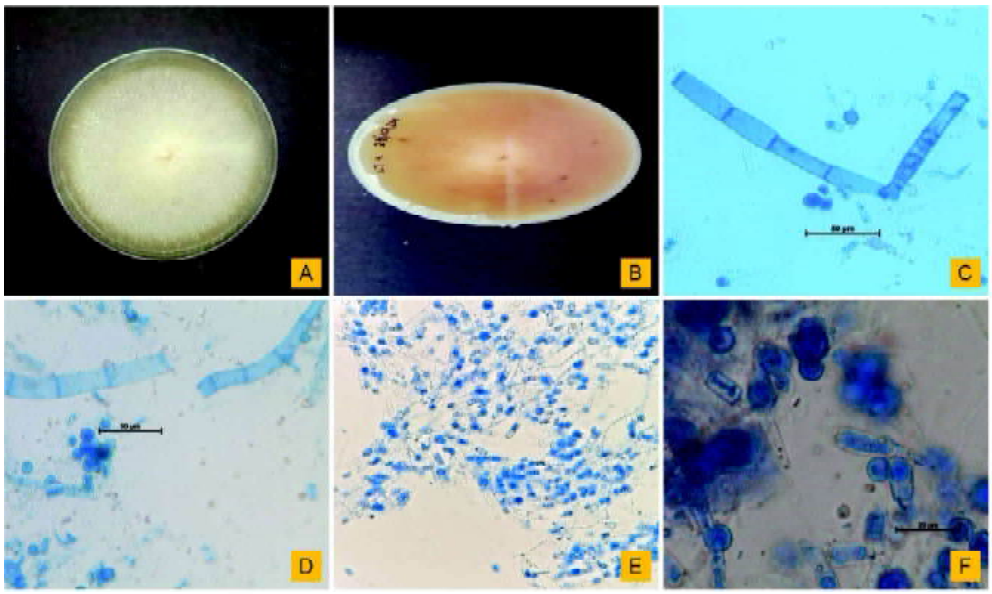

Fig. 3A-F. Fungal colony of 25B112_013_LT_1 fungal isolate in *Luisia trichorhiza*: A, Colony morphology on PDA after 14 days showing white, cottony growth; B Reverse of colony with light brown pigmentation; C–D, Septate hyphae and formation of thick-walled chlamydospores (40x); E, Abundant microconidia and chlamydospores dispersed in hyphal network (40x); F, Microconidia (hyaline, oval) arising from short phialides along with globose, thick-walled chlamydospores (100x).

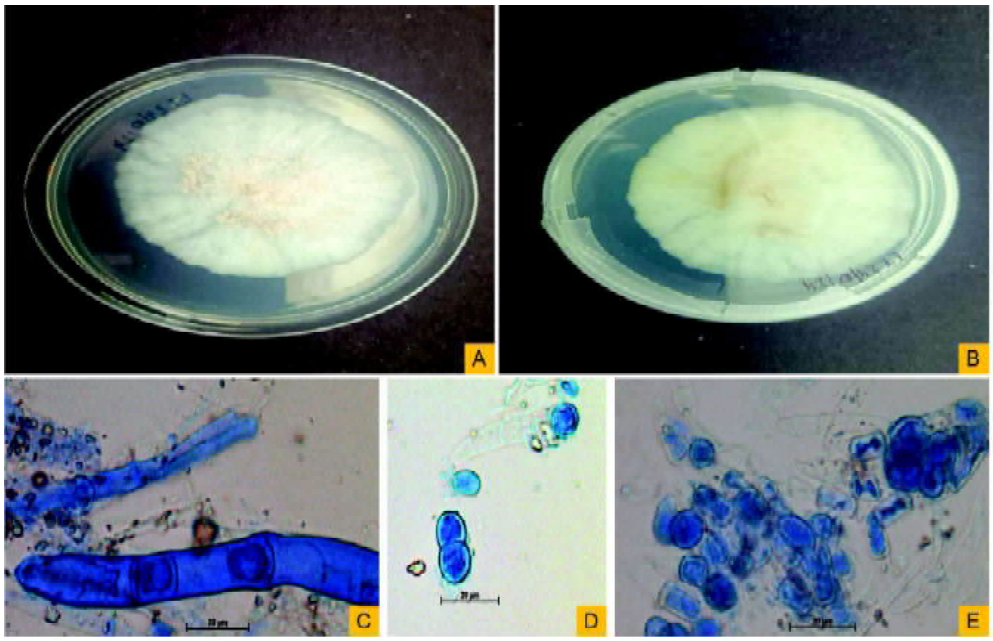

Fig. 4A-E. Fungal colony of 25B112_014_LT_2 fungal isolate in *Luisia trichorhiza*: A, 2 week-old mature colony of the fungal endophyte on PDA medium (front view), showing a cottony, dense, white mycelial growth with slightly raised central region; B, Colony exhibiting a pale-yellowish to light-brown pigmentation in the center (reverse view); C, Septate hyphae with characteristic monilioid cells observed under lactophenol cotton blue stain (100x); D, Globose to subglobose chlamydospore-like structures, single or in small clusters, with thick walls (100x); E, Aggregated chains of chlamydospore-like structures, densely packed within the hyphal matrix (100x).

zonation (Fig. 3A-B). Microscopic examination revealed septate hyphae measuring 2-4 µm in diameter, irregularly branched, and associated with the production of conidia and monilioid cells (Fig. 3C-F). In contrast, isolate 25B112_014 (LT-2) produced colonies characterized by dense aerial mycelium, distinct concentric zonation, and yellowish-brown pigmentation on the reverse (Fig. 4A-B). Microscopic observations revealed septate hyphae 3-5 µm in diameter, prolific conidial development, thick-walled chlamydospores, and multinucleate hyphae (Fig. 4C-E). The comparative assessment of colony architecture, pigmentation, sporulation structures, and specialized propagules underscores the diagnostic

Table 1. Morphological (Classical) characterization of fungal isolates.

Colony characters	Fungal isolates 25B112 013 (LT 1)	25B112 014 (LT 2)
Colour of colony (surface)	White fluffy colony	White colony with cottony growth
Colour of colony (reversed)	Brown	Yellowish brown
Shape of colony	Circular	Circular with zonation
Texture of fungal colony(Colony appearance)	Cottony with floccose texture	Cottony, slightly raised
Sclerotia present/absent	Absent	Absent
Colour changes in substratum	Light brown pigmentation	Yellow pigment diffused
Number of concentric rings	Not distinct	Concentric rings visible
Microscopic characters	25B112 013 (LT 1)	25B112 014 (LT 2)
Size	Septate variable size	Large ellipsoidal spores
Septation	Septate hyphae	Septate hypahe
Diameter of hyphae	2–4 µm approx.	3-5 µm approx.
Branching of hyphae(Pattern of branching)	Branched irregularly	Banched irregularly
Formation of rhizomorphs	Absent	Absent
Scerotia (size and texture)	Absent	Absent
Sporulation (if any)	Conidia present	Conidia and spores abundant
Characters of hyphae and spores chlamydospores	Septate hyphae + conidia	Hyphae with conidia +
Moniliod cells	Present	Present
Nuclear condition	Multinucleate	Multinucleate

separation of the two taxa. These results are consistent with established taxonomic frameworks for orchid-associated fungi, wherein cultural morphology and reproductive features constitute primary discriminants for preliminary classification (Dearnley, 2007; Otero *et al*., 2002; Rasmussen, 2002) (Table 1).

Isolation and Phylogenetic Characterization of Endophytic Fungi

The isolated endophytic fungi from surface-sterilised roots of *L. trichorhiza*, were carefully processed to obtain uncontaminated fungal cultures, and well-isolated colonies were selected for molecular characterization.

Molecular Identification and Phylogenetic Analysis

DNA sequencing has demonstrated efficacy in delineating fungal taxa (Bayman and Otero, 2006). Molecular identification was performed using sequencing of the ITS region. PCR amplification using universal ITS primers produced distinct amplicons of approximately 600 bp in both fungal isolates. The resulting sequences were analysed using BLASTn against the UNITE 9.0 and NCBI databases (Abarenka *et al*., 2010; Altschul *et al*., 1990). Isolate 25B112_013 (LT-1) exhibited 99.58% sequence similarity with *Neocosmospora rubicola* (accession KM231800), confirming its close taxonomic affiliation. The corresponding ITS sequence of LT-1 was deposited in NCBI GenBank under accession number PX640217 (Table 2). Phylogenetic analysis was conducted using the Maximum Likelihood method (Tamura *et al*., 2021) implemented in IQ-TREE (v2.1.2) with the Kimura substitution model and 1000 bootstrap replicates (Nguyen *et al*., 2015). The resulting phylogenetic tree clustered isolate LT-1 within a well-supported *Neocosmospora rubicola* clade, clearly separated from other related *Fusarium* taxa, thereby validating its molecular identification (Fig. 5).

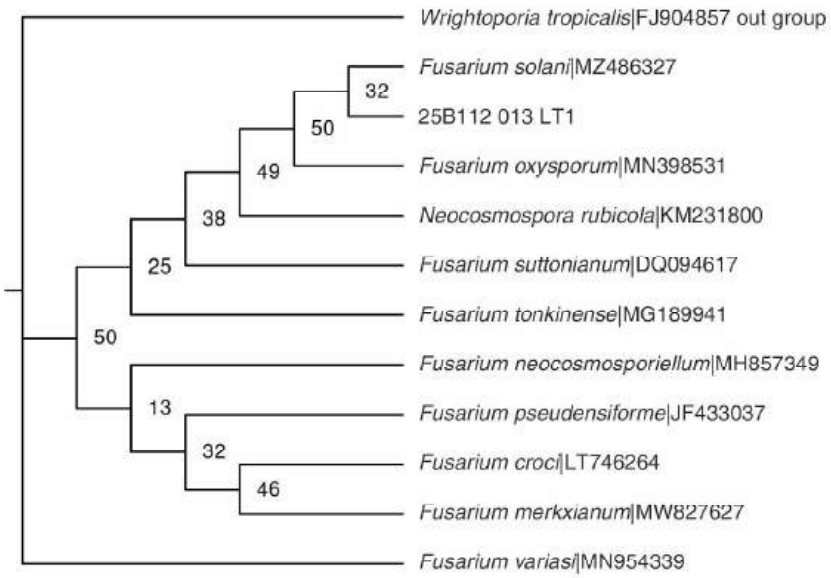

Fig. 5. Phylogenetic tree of endophytic fungal isolate 25B112 013 LT 1 in *Luisia trichorhiza*.

In contrast, isolate 25B112_014 (LT-2) showed 94.76% sequence similarity with *Thelonectria blackeriella* (accession KX778711). As this similarity value falls below the accepted threshold for confident species-level identification, the isolate was conservatively assigned to *Thelonectria* sp. The ITS sequence of LT-2 was deposited in NCBI GenBank under accession number PX735845 (Table 2). Phylogenetic reconstruction placed LT-2 within the *Thelonectria* lineage, supporting its generic placement and indicating its evolutionary relationship with other members of the genus (Fig. 6).

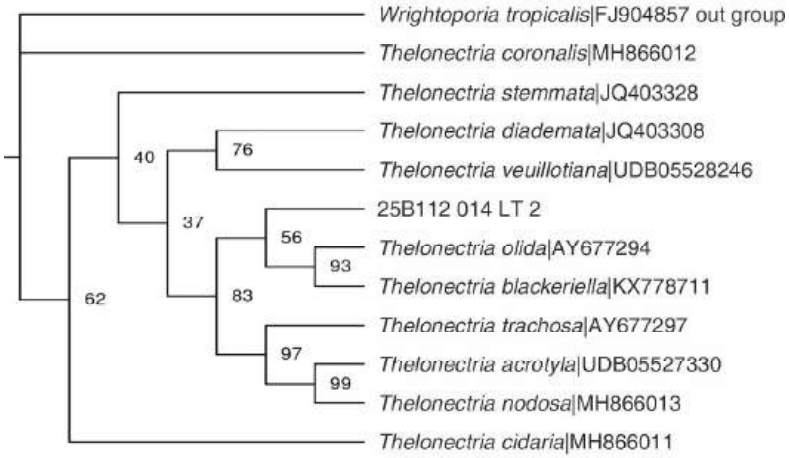

Fig. 6. Phylogenetic tree of endophytic fungal isolate 25B112 014 LT 2 in *Luisia trichorhiza*.

Table 2. Molecular identification of fungal endophytes isolated from *Luisia trichorhiza*.

Isolate	Closest taxonomic affiliation	ITS similarity (%)	GenBank accession number
LT-1	*Neocosmospora rubicola*	99.58	PX640217
LT-2	*Thelonectria* sp.	94.76	PX735845

The molecular results demonstrated that the roots of *L. trichorhiza* harbour diverse fungal taxa belonging to both *Neocosmospora* and *Thelonectria*. Similar observations of *Fusarium–Neocosmospora* complexes as common orchid-associated endophytes have been reported earlier by Behera *et al*. (2013), Deng *et al*. (2019), and Vaz *et al*. (2009). The identification of *Neocosmospora rubicola* as an endophyte is consistent with previous reports from *Bletilla striata* (Deng *et al*., 2019) and *Vanda tessellata* (Hossain, 2022), as well as studies highlighting the dominance of *Fusarium*-like taxa in orchid roots across diverse habitats (Bayman and Otero, 2006; Zettler *et al*., 2017). Reports of *Thelonectria*-like fungi from orchids remain scarce; however, comparable diversity of ascomycetous endophytes has been documented from orchids such as *Cymbidium* (Yuan *et al*., 2009), *Dendrobium* (Xing *et al*., 2011), *Vanilla planifolia* (Gazis and Chaverri, 2010), and various other orchids (Bukhari and Velip, 2024; Lekshmi and Decruse, 2023). The recovery of such relatively uncommon taxa suggests that orchids may harbour a broader and more complex fungal community than the previously recognised one (Delaye *et al*., 2013; Varma *et al*., 2017). The coexistence of dominant and rare fungal associates further indicates that the root microbiome of *L. trichorhiza* is shaped by a balance of functional partners, which may enhance ecological resilience and adaptability (Givnish *et al*., 2016; Waterman and Bidartondo, 2008). Such associations may play an important role in orchid survival and may offer promising avenues for biotechnological applications in plant growth promotion, secondary metabolite production, and conservation of RET (rare, endangered, and threatened) orchid taxa as also suggested earlier by Chua *et al*. (2022) and Strobel and Daisy (2003).

Conclusion

During the present investigation, identification and characterization of two endophytic fungi (*Neocosmospora rubicola* and *Thelonectria blackeriella*) which were found to be associated with an epiphytic species, *L. trichorhiza* may add further to the knowledge of orchid-associated fungi. These endophytes may play a role in nutrient uptake and stress tolerance and hence, the present results establishes a baseline for future efforts in orchid conservation, *in vitro* symbiotic germination, and bioprospecting.

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