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ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI FROM THREE VANDACEOUS ORCHID SPECIES OF WESTERN GHATS, INDIA

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Abstract

Orchids generally require a symbiotic fungus to complete their life cycle and thus characterization and identification of endomycorrhiza is essential. The most common mycorrhizal fungi of orchids belong to the genera *Ceratobasidium*, *Sebacina*, *Thanetophorus*, and *Tulasnella* of Basidiomycota. The present study describes endophytic fungi from the roots of *Papilionanthe subulata* (Willd.) Garay, *Rhynchostylis retusa* (L.) Blume, and *Taprobanea spathulata* (L.) Christ, the three epiphytic orchids of Western Ghats. Five endophytic fungi were isolated from anchored roots, characterized morphologically and designated as Vs1, Vs2, Rr, Ps1, and Ps2. They were morphologically similar to that of the form genus *Rhizoctonia* and were binucleate in their vegetative hyphae and monilioid cells. Sequencing of ITS region and phylogenetic analysis revealed that Rr, VS1, and VS2 have close similarity to *Ceratobasidium* sp. while Ps1 and Ps2 with *Tulasnella*. Based on sequence similarity, Vs1 and Vs2 were assigned the species status *Ceratobasidium lantanae-camarae* and *C. gomesae* respectively and are new report from India. Other three isolates did not clad with any described species having sequences in the NCBI database.The sequences of the isolates were deposited in NCBI Genbank with the accession numbers, Rr_ITS OL374043, VS1_ITSOL374050, VS2_ITSOL374052, PS1_ITSOL374163, and Ps2_ITSOL374168 respectively. The identified isolates are members of Ceratobasidiaceae or Tulasnellaceae and thus have symbiotic potential for further characterization and evaluation.

Introduction

Orchids have minute seeds with small embryos and without endosperm (cf. Arditti, 1992). They entirely depend on appropriate mycorrhizal fungus for their nutrient and water supply during seed germination, seedling development, and establishment with a long-term survival rate. Therefore, the presence of mycorrhizae is essential for successful colonization and establishment of an orchid in natural, as well as managed or restored habitats (Leake, 1994; Rasmussen, 2002; Zettler, 1997). Kaushik (1983) studied mycorrhiza in 53 species of orchids which included both terrestrial and epiphytic orchids and found intracellular potentials and intercellular hyphal filaments in cortical cells of transections of roots and intracellular hyphal in longitudinal sections of roots. Though, about 69% of orchid species have an epiphytic lifestyle on trees (Zotz, 2013), mycorrhizal communities associated with epiphytic orchids have been investigated less frequently as compared to the terrestrial orchids (Dearnaley *et al.,* 2012; Rasmussen, 2002; Yukawa *et al.,* 2009). As per early reports, epiphytic orchids have a low intensity of infection with mycorrhizal fungus as compared to terrestrial species (Hadley and Williamson, 1972). The protocorms of epiphytic orchids often become photosynthetic at an early stage and the roots of adult plants have very little and sporadic fungal colonization. Thus, the degree of mycorrhizal association is reported to be less clear in epiphytic orchids (cf. Arditti, 1992). Aerial roots in epiphytic species are generally considered devoid of mycorrhizal infection but the roots in contact with the substrate are extensively infected (Goh *et al*., 1992). Thus, the orchids growing on trees obtain adequate mineral nutrients from dust, organic debris, and stem-flow along with the bark of the host. The unstable availability of water and nutrients in their arboreal habitats often result in severe water and nutrient stresses. Consequently, mycorrhizal associations may be important for the survival of epiphytic orchids (Martos *et al*., 2012; Rammitsu *et al*., 2020). Germinating epiphytic orchid seeds are also documented to obtain water through mycorrhizal fungi (Yoder *et al*., 2000) which help in sustaining their long-term survival. The sustainability of epiphytic orchids is thus influenced by the presence of orchid mycorrhizal fungi.

The common fungi of orchid mycorrhizae are the members of Ceratobasidiaceae, Tulasnellaceae, and Sebacinaceae of Basidiomycota (Dearnaley *et al*., 2012; Wells, 1994). The mycorrhizal community of the epiphytic orchids is strongly biased toward a single Ceratobasidiaceae fungus despite a wide range of fungal partners in its entire distribution range as reported in *Thrixspermum japonicum* (Rammistu *et al.,* 2020). The Ceratobasidiaceae members are mostly species of *Ceratobasidium* as in *Cymbidium goeringii* (Wu *et al*., 2010), *Dendrobiuum formosum* (Khamchatra *et al*., 2015), *Rhynchostylis retusa* (Bhuiyan *et al*., 2021), *Vanda spathulata* (Lekshmi and Decruse, 2018),

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V. thawaitesii (Decruse *et al.,* 2018), and *Thanetophorous* as in *V. coerulea* (Aggarwal *et al*., 2012). Species of Tulasnellaceae are also known to support symbiotic activity in the epiphytic orchids; *Coelogyne nervosa* (Sathiyadash *et al*., 2014), *Dendrobium aphyllum* (Zi *et al*., 2014), *Grammattophyllum speciosum* (Nontachaiyapoom *et al*., 2011), and *Paphiopedilum villosum* (Khamchatra *et al*., 2016). Kaushik and Pal (2011) described pathogenicity in other plants but mycorrhizal association with orchids. The fungi are known to provide phosphate, minerals, vitamins, exogenous carbon sources, and other growth factors. Lignin degrading capacity of mycorrhizal fungus *Rhizoctania solani* was assessed by Kaushik and Pal (2012) which was isolated from *Vanda testacea*. However, the Western Ghat orchids are least explored with regard to mycorrhizal diversity and thus three vandaceous orchids of Western Ghats namely *Papilionanthe subulata* (Willd.) Garay, *Rhynchostylis retusa* (L.) Blume, and *Taprobanea spathulata* (L.) Christ has been subjected to investigation to understand their mycorrhizal association as it is important for their conservation. *Taprobanea spathulata,* though is an epiphytic orchid, starts its life cycle as a terrestrial species; it has large endemic distribution in Southern India and in Sri Lanka. The species is considered as *Vulnerable* (VU) as per IUCN categorization (Chadburn and Khela, 2014). *Rhynchostylis retusa* is kept under *Endangered* category under Appendix II (with strictly controlled international trade) of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) by the Government of India, due to decline in its populations in wild (Das *et al*., 2013). *Papilionanthe subulata* is another vandaceous orchid endemic to Kerala, Tamil Nadu, and Karnataka States in India and Sri Lanka.

Material and Methods

Plant Material

Papilionanthe subulata (Willd.) Garay, *Rhynchostylis retusa* (L.) Blume, and *Taprobanea spathulata* (L.) Christ were the species selected for the present study. *P. subulata* attached to twigs of host (*Careya arborea*) in its native locality (Chathurangappara: 9°47'58.00" N-77°13'15.84" E) in Idukki district of Kerala was collected and the roots attached to the twigs were used for the study. Roots of *R. retusa* adult plants attached to bark of its host (Teak) from native locality (Ranni: 9°23'8.19" N -76°49'31.02" E) in Pathanamthitta District of Kerala was used for the study. Roots of *T. spathulata* embedded in soil were collected from adult plants growing on rocks in a native locality (Amboori: 8°30'19.22" N-77°10'27.26" E) in Thiruvananthapuram district of Kerala, India.

Isolation of Mycorrhizal Fungi

Single peloton method (Zhu *et al*., 2008) was used to isolate mycorrhizal fungi. Actively growing roots from the plants in their native localities were collected and wrapped in wet newspaper and brought to the laboratory in the same or next day. The roots were washed in running tap water to remove bark and adhering debris, surface-sterilized in 5% sodium hypochlorite solution for 10 min followed by dipping in spirit and flaming twice. Subsequently, the root segments were washed in several changes of sterile distilled water and then cut into 1-2 cm segments using a sterile razor blade followed by peeling of the root epidermis and teasing out the pelotons from cortical cells using sterile needles in a few drops of sterile distilled water. The pelotons released out from the root cortex were washed thoroughly in sterile distilled water and then diluted to get 2-3 pelotons in a drop. A few drops of diluted pelotons were transferred to 80 mm glass Petri plates containing 15 ml pre-sterilized fungal isolation medium (FIM: Clements *et al*., 1986; Hollick, 2004). The plates were incubated at ambient conditions (25±3°C) for 3-7 days. Single tips separated from the fungal hyphae emerging from the pelotons were sub-cultured on FIM containing tetracycline (50 mgl^{-1}) and streptomycin sulfate (100 mgl^{-1}) as antibiotics. This step was repeated 2-3 times to confirm that the isolate is from a single strain and free of bacterial contamination. Further subculture was made in $1/5^{th}$ strength of Potato Dextrose Agar (PDA) medium. Fungi showing different growth characteristics were purified similarly and maintained in $1/5^{th}$ strength of PDA, at 25±3°C with monthly subculture.

Characterization

Morphological Characterization

Morphological characterization was done based on outlined methods (Zelmer *et al.,* 1996). The colony colour, radius, mycelial zonation, monilioid cells, thickness of hyphae, branching patterns, and growth rate were analyzed under a phase-contrast microscope. Stained (Safranin or Lactophenol Cotton Blue) images of hyphae were taken under a compound microscope with an attached camera.

Cytological Characterization

Safranin-O- KOH method (Bandoni, 1979) of nucleolar staining was undertaken to study nuclear status of the isolated fungi. Both vegetative hyphae and monilioid

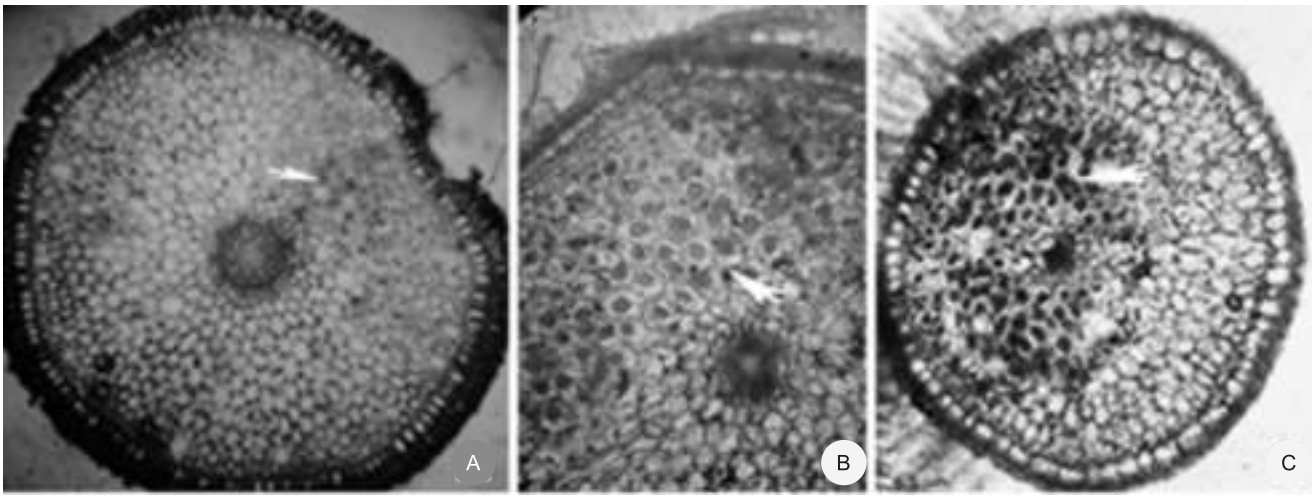

Fig. 1. Cross sections of roots showing mycorrhizal colonization in the cortical cells: A, *Taprobanea spathulata*; B, *Rhynchostylis retusa*; C, *Papilionanthe subulata.*

cells were examined under a light microscope and photographic images were captured.

Molecular Characterization

Molecular characterization was done by isolating genomic DNA and sequencing the ITS region. It was outsourced and performed at Omicsgen Pvt Ltd, Kochi, Kerala, India. DNA was isolated from actively growing culture, electrophoresed in 1% Agarose gel, ITS region PCR amplified with specific primers (ITS1 and ITS4) and amplicon was checked for appropriate size by Agarose gel visualization. Amplicon was gel purified using commercial column-based purification kit (Invitrogen, USA) and sequencing was performed with forward and reverse primers in ABI 3730 XL cycle Sequencer. Forward and reverse sequences were assembled and contig was generated. An online tool BLAST of NCBI database were performed for sequence analysis and based on the maximum identity score E value, top most sequence was utilized for multiple sequence alignment. The evolutionary history was inferred using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993) and the optimal tree was generated.

Culture Deposits

The cultures of all the isolates were deposited with Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh and accession numbers were obtained.

Results

Morphological Characterization

Root samples of *Papilionanthe subulata* (Willd.) Garay, *Rhynchostylis retusa* (L.) Blume, and *Taprobanea spathulata* (L.) Christ collected from their native localities possessed high mycorrhizal colonization in the cortical region (Fig. 1). Culture of pelotons and root segments resulted in the outgrowth of several endophytic fungi, of which two (designated as Ps1 and Ps2) from *P. subulata,* one (Rr) from *R. retusa*, and two (Vs1 and Vs2) from *T. spathulata* were *Rhizoctonia*

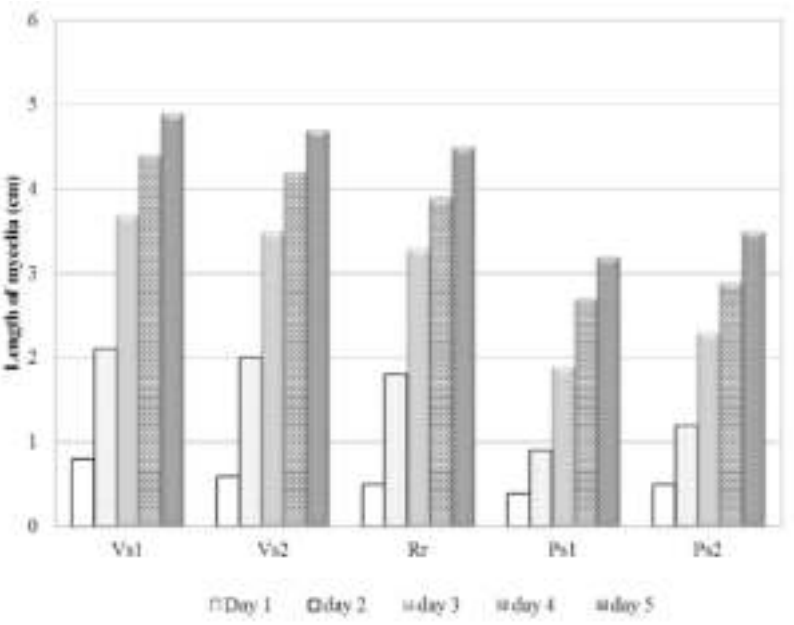

Fig. 2. Growth rate of fungal isolates on 1/5th strength PDA medium.

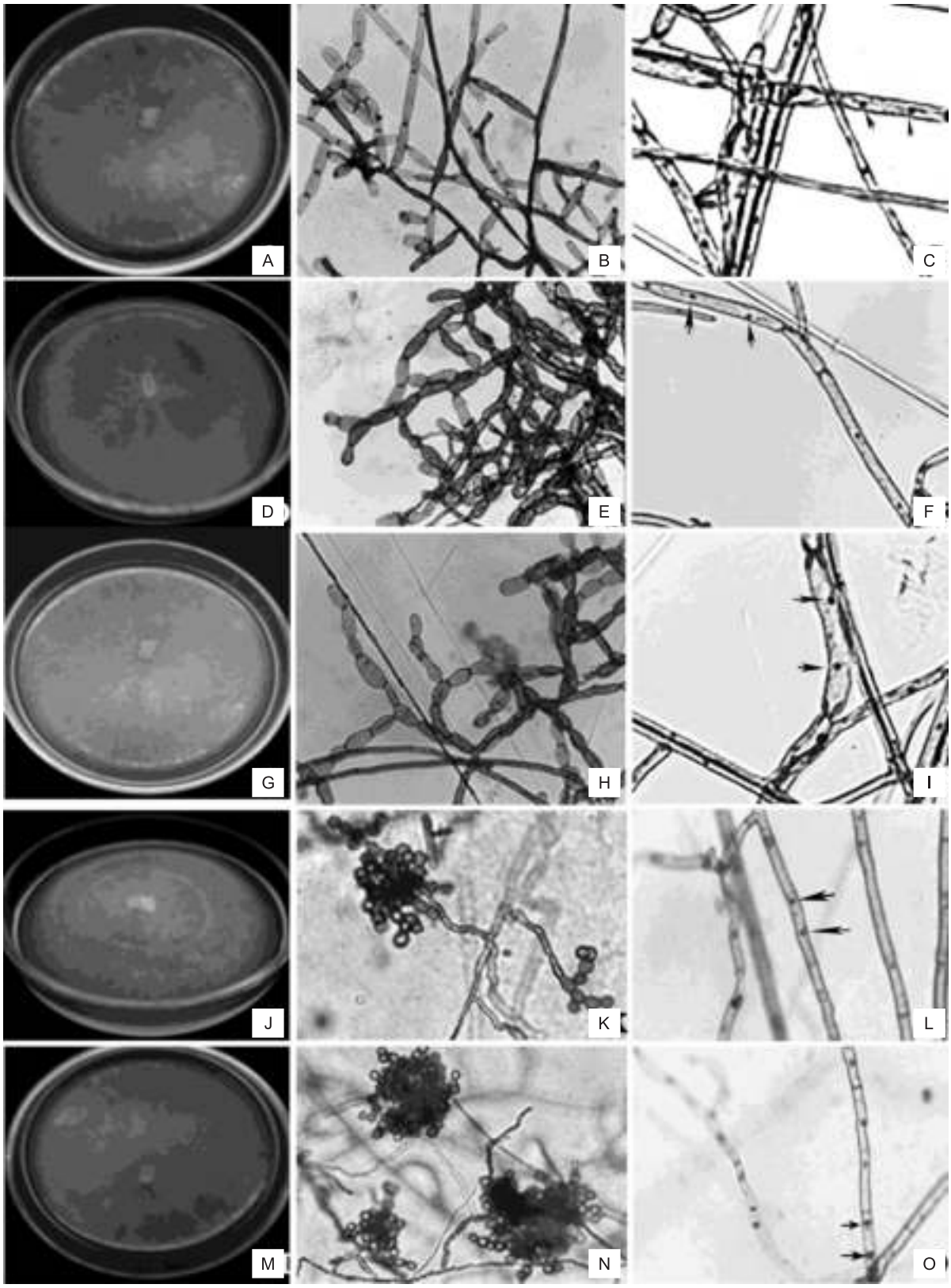

Fig 3. Endophytic fungi isolated from the roots of 3 orchid species: A-C, *Ceratobasidium*_VS1 from *T. spathulata*; D-F, *Ceratobasidium*_VS2 from *T. spathulata*; G-I, *Ceratobasidium*_Rr from *R. retusa*; J-L, *Epulorrhiza*_PS1 from *P. subulata*; M-O, *Tulasnella*_PS2 from *P. subulata*; A,D,G,J,M, Colony morphology; B,E,H,K,N, monilioid cells; C,F,I,L,O, vegetative hyphae showing binucleate.

like and further characterized. These five isolates showed differential growth pattern in 1/5th strength of PDA medium (Table 1; Fig. 2). The fungal strain Vs1 showed exact right- angled pattern of branching, pale white colony, and attained 4.9 cm radial growth within 10 days of inoculation (Table 1; Fig. 3A). It produced long barrel shaped monilioid cells in small clusters within 6-8 days (Fig. 3B). The other strain from *T. spathulata* (Vs2), showed white colour colony and attained 4.7 cm radius in 8-10 days (Fig. 3D). It also produced long elliptical to barrel shaped monilioid cells, frequently within 6-8 days (Fig. 3E). The fungal strain Rr from *R. retusa*, showed approximate right-angled branching pattern, slightly white cottony colony, and attained 4.5 cm radial growth in 10 days (Fig. 3G). The monilioid cells were produced in long chains in 9-10 days and the cells were long elliptical shaped (Fig. 3H). Both Ps1 and Ps2 were isolated from *P. subulata*, with very thin mycelia. Ps1 appeared as pale white colony and made strict zonation (Fig. 3J) and produced small round monilioid cells in small clusters (Fig. 3K) and Ps2 appeared as pale white transparent colony, attained 3.5 cm radial growth in 10 days (Fig. 3M), and produced small round monilioid cells frequently in large clusters (Fig. 3N). Both Ps1 and Ps2 colonies appeared as rubbery or leathery in texture.

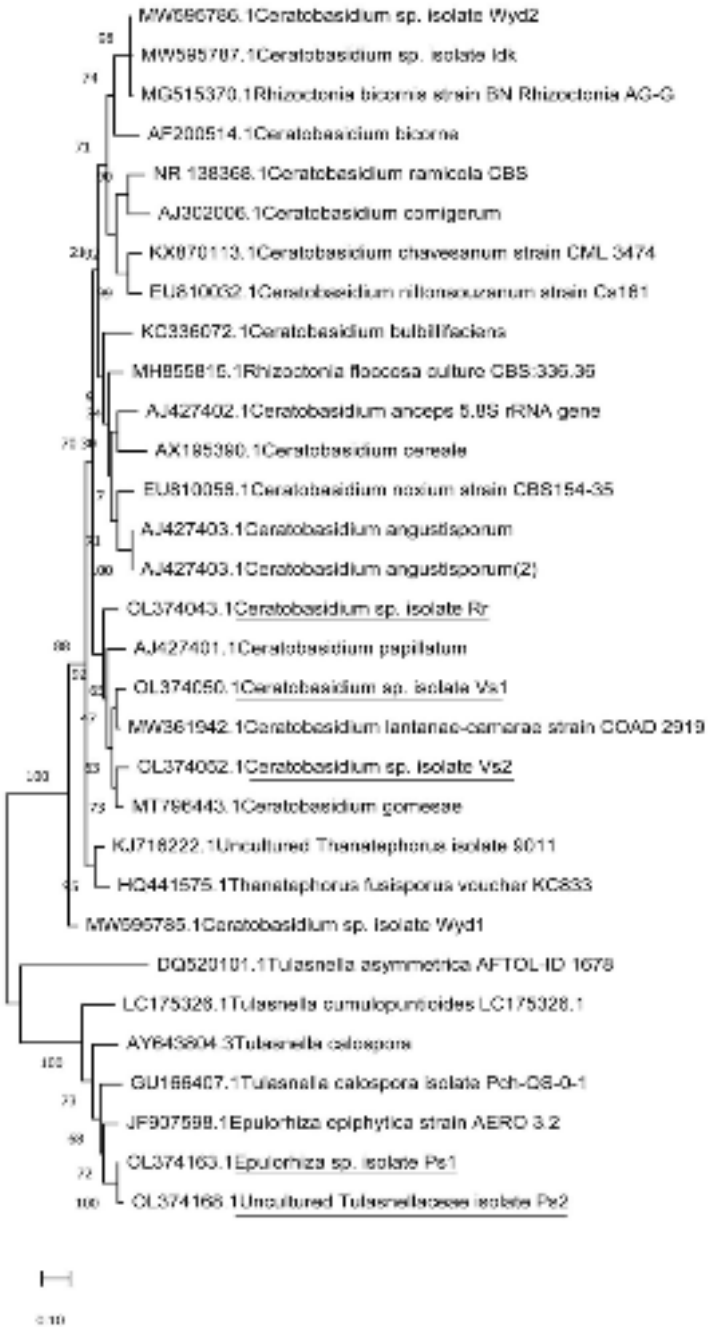

Fig. 4. Phylogenetic tree showing the phylogenetic relations of fungal isolates.

Cytological Characterization

The mycelial cells of all the five isolates were binucleate as revealed by nucleoli staining (Fig. 3C, F, I, L, O).

Molecular Characterization

Molecular identification through sequencing the internal transcribed spacer (ITS) regions of the rRNA gene revealed (Table 2) that Vs1 have 96.72% sequence similarity with *Ceratobasidium lantanae-camarae* (GenBank: MW361942), Vs2 have 96.98% sequence similarity with *Ceratobasidium* gomesae (GenBank: MT796440), Rr have 95.59% similarity with a *Ceratobasidium* sp. M-13 (JQ713569) and at species level the close match is *C. papillatum* with 90.27% sequence similarity. Ps1 showed 99.83% sequence similarity with *Epulorhiza* sp. (AJ 313438) and Ps2 have 98.68% identity with an uncultured Tulasnellaceae clone (MH005882). Both the isolates have 98.77% sequence similarity but their close match at species level is *Epulorhiza epiphytica* with 92.61 and 91.42% similarity, respectively. The sequence was deposited with NCBI database and accession numbers were assigned as *Ceratobasidium* sp. isolate Vs1 OL374050, *Ceratobasidium* sp. isolate Vs2 OL374052, *Ceratobasidium* sp. isolate Rr OL374043, *Epulorhiza* sp. isolate Ps1 OL374163, and Uncultured Tulasnellaceae isolate Ps2 OL374168, respectively for VS1, VS2, Rr, PS1, and PS2. Phylogenetic tree (Fig. 4) generated based on the sequences also revealed the affinity of the three isolates Vs1, Vs2, and Rr in a single cluster with

Table 1. Morphological characteristics of fungal isolates grown on 1/5th strength PDA (Potato Dextrose Agar) medium for 7 days.

Fungal strains	Thickness of mycelia (µm, mean ±SD) / Branching pattern	Colony colour	Presence of monilioid cells	Shape and size of monilioid cells	Number of monilioid cells in a chain	Colony zonation	Nuclear status
Vs1	2.96± 0.15 thick/ Exact right angled	Pale White	Small clusters	Long barrel (6.33±1.66 µm wide, 10.53±2.68 µm long)	Short chain, 4-8 cells	No	Binucleate
Vs2	2.13± 0.05 thick/ right angled	Pale white	Yes	Long barrel (6.26±0.63 µm wide, 9.86± 1.43 µm long)	Long chain, 6- 11	No	Binucleate
Rr	2.5 ± 0.34 thick/ right angled	White, Cottony growth	Yes	Long elliptical (4.63± 0.85 µm wide, 8.13 ± 1.10 µm long)	Long chain, 8-13 cells	No	Binucleate
Ps1	Very thin (1.8±0.1)/ slightly right angled	Pale white,	Small clusters	Small, round (2.4± 0.1 µm wide, 3.43±0.66 µm long)	Short chain, 6-8 cells	Strict zones	Binucleate
Ps2	Very thin(1.43±0.23)/ highly branched	Pale white, transparent	Large clusters	Small, round (2.86±0.20 µm wide 3.43± 0.28 µm long)	Short chain, 6-9 cells	No	Binucleate

Ceratobasidium sp. while Ps1 and Ps2 appeared in a different cluster with Tulasnellaceae members.

Culture Deposits

The cultures of all the isolates were deposited with Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh and obtained accession numbers as Vs1-*Ceratobasidium lantanae-camarae* (MTCC 13377), Vs2- *Ceratobasidium gomesae* (MTCC 13378), Rr- *Ceratobasidium* sp. (MTCC 13379), Ps1- *Tulasnella* sp. (MTCC 13381), and Ps2- *Tulasnella* sp. (MTCC 13382).

Discussion

Colonization by suitable fungi in the root cortex is an established fact to meet the high nutritional demand for vigorous vegetative growth of orchids (Masuhara and Kutsuya, 1992; Shagufta *et al*., 1993). The pelotons in the inner cortex may have a brownish colour and loose hyphal coils because the host's lytic enzymes have digested the fungal mass to release nutrients. The moderate defense mechanisms possessed by orchids to control the level of fungal infection are through the production of phytoalexins (Rasmussen and Whigham, 2002). The roots procured from the presently investigated species namely, *P. subulata*, *R. retusa*, and *T. spathulata* with luxuriant vegetative growth exhibited high colonization of endomycorrhizae, in the cortical cells as evidenced from the presence of pelotons. This is probably having relevance in supporting the growth of the orchids in the native habitat exposed to adverse environmental conditions.

The peloton formation and monilioid cells (asexual resistant propagules) development are the common features for *Rhizoctonia*-like orchid endophytes (Rasmussen and Whigham, 2002; Shan *et al*., 2002).

Table 2. Phylogenic relations of fungal isolates.

Fungal isolates	Host	ITS sequence length	Phylogenetic relationship Close match in Genbank (Accession No.)	Maximum Identity
Vs1	*T. spathulata*	520	*Ceratobasidium lantanae-camarae* (MW361942)	96.72%
Vs2	*T. spathulata*	551	*Ceratobasidium gomesae* (MT796440)	96.98%
Rr	*R. retusa*	524	*Ceratobasidium* sp. M- 13 (JQ713569)	95.59%
Ps1	*P. subulata*	581	*Epulorrhiza* sp. (AJ 313438) *Epulorhiza epiphytica* strain AERO_3.2 (Accn No.JF907598)	99.83% 92.61%
Ps2	*P. subulata*	604	Uncultured Tulasnellaceae clone (MH005882) *Epulorhiza epiphytica* strain AERO_3.2 (JF907598)	98.68% 91.42%

Ceratobasidium sp. in common produces ellipsoidal to barrel shaped monilioid cells in culture (Khamchatra *et al*., 2016; Steinfort *et al.,* 2010) and have thick hyphae with binucleate cells (Garcia *et al.,* 2006; Hossain *et al*., 2013; Steinfort *et al.,* 2010). Presently, the fungal strains Vs1 and Vs2 produced long barrel shaped monilioid cells whereas Rr produced elliptical to barrel shaped monilioid cells, in cultures. Another crucial characteristic for identifying *Rhizoctonia*-like fungus is their variable nuclear number per cell, which ranges from uninucleate to binucleate to multinucleate in different orchid mycorrhizal fungi and even in different strains of the same species (Otero *et al*., 2002). Literature studies revealed that fungi in *Rhizoctania* complex can be divided into two groups *i.e.* multinucleate and binucleate taxa (Kaushik and Pal, 2011). Uninucleate *Rhizoctonia*-like fungi are considered pathogenic for other plants and are rarely found in orchids (Otero *et al*., 2002; Rasmussen and Whigham, 2002). Multinucleate *Rhizoctonia*-like fungi were also reported in orchids (Carling *et al*., 1999; Hossain, 2019). The endophytes investigated here possessed two nuclei in vegetative and monilioid cells and thus confirmed their presence to the binucleate group of *Rhizoctonia*-like fungi which are reportedly most common and broadly distributed in orchids (Otero *et al*., 2002). The above mentioned morphological characters of Vs1, Vs2, and Rr are closely related to species belonging to the genus *Ceratobasidium*. One of the most prevalent and peculiar genera that forms mycorrhizas with terrestrial orchids is Tulasnella/*Epulorhiza* (Currah and Zelmer, 1992) and it is considered to be a less common mycorrhizal partner of tropical epiphytic orchids (Richardson and Currah, 1995; Richardson *et al*., 1993). *Epulorhiza* is recognized by hyaline hyphae with constricted branch points, binucleate, globose or irregular monilioid cells, creamy white to pale tan or orange colony, rubbery or leathery in appearance and texture (Currah and Zelmer, 1992; Currah *et al*., 1997; Zelmer and Currah, 1997). The two fungal strains Ps1 and Ps2 from *P. subulata* isolated and characterized in this study resembled to those described for *Epulorhiza*/*Tulasnella* with thin hyphae, small globose or spherical monilioid cells, pale transparent colony and produce binucleate cells and thus probably belong to the genus *Epulorhiza/Tulasnella*. Though, sexual spore formation is essential for the confirmation of species identity, molecular methods through sequencing of ITS region of ribosomal RNA are widely accepted as a tool to find close resemblance to an already described species or isolates. Specimen with a sequence similarity of 97% or above are considered to be a single species but is not a hard and fast rule (Johnson *et al*., 2019). This number is highly variable between different groups and thus a definitive percentage sequence similarity that could precisely indicate conspecific taxa is lacking. Consequently, no single cut off value has been universally established for species identification across the kingdom fungi. Therefore, based on the findings of the GenBank BLAST search, it is fair to begin with 80% query coverage and 97-100% sequence similarity when awarding a species name (Raja *et al.,* 2017) as the average weighted intraspecific ITS variability in the kingdom fungi was calculated to be 2.51%. The estimate was 3.33 for Basidiomycota (Raja *et al.,* 2017) and thus sequence similarity of 96.67 and above may be considered as same species of Basidiomycetes are based on the current understandings.

In the present study, the fungal isolate Vs1 showed 96.72% sequence similarity with *Ceratobasidium lantanae-camarae* strain (MW361942) at species level. *Ceratobasidium* sp. isolate Vs2 (OL374052) is closely related to *Ceratobasidium gomesae* (MT796439.1) with 96.98% sequence similarity. As reviewed by Raja *et al.* (2017), there is 3.33% divergence on sequences of Basidiomycota. Hence, the Vs1 and Vs2, are assigned the species status as *C. lantanae-camarae* and *C. gomesae*. Their close affinity is also evident in the Phylogenetic tree (Fig. 4). As the 3.33% sequence divergence may vary with new sequence deposits, the species name assigned is only based on present understanding. *C. lantanae-camarae* H. C. Evans, R. W. Barreto & C. A. Ellison *sp. nov*. is originally reported from Brazil as a pathogen causing white thread blight disease of *Lantana camara* (Barreto *et al.,* 1995). It was later synonymized as *C. cornigerum* but based on molecular analysis, the name was reinstated by Ferreira *et al.* (2021).This is the first time a close match of the species is reported as an orchid endophyte and is the first report from India. *C. gomesae* E.S. Cruz, Meir. Silva, M.C.M. Kasuya, is an endophyte from the epiphytic orchid *Gomesa recurva* endemic to Brazil and Argentina. It is described exclusively from cultures (Anamorph), through morphological and blast analysis (Cruz *et al.,* 2022). This is the first report of the species from India from an orchid *T. spathulata* growing as a lithophyte in Thiruvananthapuram district of Kerala,

Ceratobasidium sp. isolate Rr (OL374043) did not clad with any described species (Fig. 4) but the nearest match is *Ceratobasidium papillatum* (AJ427401.1) with 90.27% sequence similarity at species level and among other isolates, *Ceratobasidium* sp. M- 13 (JQ713569), with 95.59% similarity. Therefore, *Ceratobasidium* sp. isolate Rr seems to be a new species. However, 70% of the described species of fungi are not sequenced (Rossman and Palm-

Hernandez, 2008) and thus the respective sequences are not available in the gene bank for blast analysis. As far as *Ceratobasidium* sp. are concerned, there are 45 species as per Kew records (http://www.indexfungorum.org/names/names.asp, accessed on 23-09-2023) and the sequences of only 20 species are available as per NCBI database (accessed on 19-09-2023).Therefore, assigning new species status based on blast analysis should be made with caution as suggested (Raja *et al*., 2017) and thus, the present knowledge is insufficient to assign a species status for *Ceratobasidium* sp. isolate Rr.

Sequence similarity of Ps1 and Ps2 is with members of Tulasnellaceae; Ps1 showing 99.83% similarity with *Epulorhiza* sp. (AJ 313438) and Ps2 showing 98.68% similarity with uncultured Tulasnellaceae clone (MH005882). However, a close match to species level could not be obtained through BLAST search but the nearest species having similarity was *Epulorhiza epiphytica*- JF907598.1 with 92.61% and 91.42% similarity for Ps1 and Ps2 respectively. The BLAST result also revealed that the sequence similarity of Ps1 & Ps2 was 98.77% indicating thereby that they are different strains of the same species. However, the discrepancy in number of reported species and ITS sequences among the databases also exists for *Tulasnella/ Epulorhiza* sp. and thus Blast analysis and the morphological data on the cultures are insufficient to assign new species status for the isolates.

Morphological characteristics have mainly been used in the taxonomy of *Tulasnella* spp. (Currah *et al.,* 1997; Pereira *et al.,* 2003; Zelmer and Currah, 1997) and molecular phylogenetic analysis used in *E. amonilioides* (Almeida *et al*., 2014). Some ecological research on orchid mycorrhizae that identified *Epulorhiza/Tulasnella* fungi using molecular data raised the possibility of the existence of unrecognized taxa (Fujimori *et al*., 2018). Most of the *Tulasnella* species reported were isolated and identified from terrestrial orchids as *Arthrochilus, Caleana, Chiloglottis, Drakaea* (Linde *et al.,* 2017), *Spathoglottis plicata* (Athipunyakom *et al.,* 2004), and *Spiranthes sinensis* (Fujimori *et al*., 2018). Existence of *Tulasnella* species in epiphytic orchids as *Cattleya cinnabarina, Dendrobium nobile, Hadrolaelia jongheana*, and *Zygopetalum maxillare* (Chen *et al*., 2012; Freitas *et al.,* 2020) is also known. *E. epiphytica* exhibiting 91% sequence similarity with the isolate Ps2 in the present study is also known from the two epiphytic orchids *Epidendrum rigidum* and *Polystachya concreta* (Pereira *et al.,* 2003). Therefore, we report here the presence of two Tulasnellaceae isolates from *P. subulata,* an epiphytic orchid of Western Ghats.

The molecular and morphological approaches established for isolation and identification of mycorrhizal fungi from *P. subulata*, *R. retusa,* and *T. spathulata* revealed the existence of undescribed *Ceratobasidium* species and *Tulasnella* species in Western Ghat orchids. Two species of *Ceratobasidium* or their close match, *C. lantanae-camarae* VS1 and *C. gomesae* Vs2 were identified from the roots of *Taprobanaea spathulata* and are new report from Indian orchids. A close match of the *Ceratobasidium* isolate Rr and *Tulasnella* isolates Ps1and Ps2 could not be obtained and thus, the present knowledge is insufficient to assign species status to them. This study will facilitate to elucidate the diversity and variability of the species of mycorrhizal fungi. This would allow for the description of novel fungal species and the application of mycorrhizal fungi in conservation efforts. As orchids are included in the list of Appendix II of CITES, there is a great need to conserve and mass propagate orchid species (Pathak *et al.,* 2022, 2023; Prakash and Pathak, 2019). Some attempts have already been made for *ex situ* conservation of a few commercially important and/or endangered orchids by some workers (Anuprabha and Pathak, 2019, 2020; Bhatti *et al.,* 2017; Bhowmik and Rahman, 2020, 2022; Kumari and Pathak, 2021; Lalduhsanga *et al.,* 2021; Lekshmi and Decruse, 2018; Mutum *et al.,* 2022; Pathak *et al*., 2022, 2023; Sembi *et al.,* 2020; Sunita *et al.,* 2021; Thakur and Pathak, 2020, 2021; Tripura *et al.,* 2022; Vasundhra *et al.,* 2019, 2021).

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