First report on isolation and characterization of endophytic Colletotrichum species from *Nervilia crociformis*, a terrestrial orchid

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ABSTRACT

Nervilia crociformis, a terrestrial orchid species, native to Southeast Asian nations, is unique for having a brownish-green colored crocodile's head-like appearance of its flowers. The present study aimed to isolate and characterize the endophytic fungi from their mycorrhizal roots. *Nervilia crociformis* and rhizosphere soil samples were collected from the Shola forests of Kemmangundi, Western Ghats, India. Mycorrhizal fungi were isolated from thin roots, pseudobulb, and tubers of *Nervilia crociformis.* Light microscopy showed hyphal colonization on the surface of roots as well as inter- and intracellular root colonization with intact and digested pelotons. Transmission electron microscopic studies found the inner layer of the internal hyphae, whereas the outer layer was more granular. Molecular characterization through phylogenetic analysis of *ITS* sequences confirmed the fungal species as *Colletotrichum fructicola.*

Keywords: Orchid, Fungi, Transmission electron microscopy, phylogenetic analysis, mycorrhiza

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1. INTRODUCTION

Nervilia Comm. ex Gaud., a genus of over 100 species of orchids, has tiny underground tubers, and an inflorescence with one to several flowers (Govaerts et al., 2018). There are 16 species known to exist in India. *Nervilia crociformis*, commonly known as the Crocodile Orchid or Crocked Nervilia, is a small terrestrial orchid species of this genus. Its unique brownish-green

colored, dark patterns on the lip make the appearance of a flower of *N. crociformis* that resembles a crocodile's head (Kanapol et al., 2019). It is found in Southeast Asian nations like Thailand, India, Malaysia, Indonesia, Singapore, and the Philippines. In India, Southern Indian states including Andhra Pradesh, Tamil Nadu, Kerala, Karnataka, and Odisha are known to have *Nervilia crociformis*. It has been noted in Assam and Meghalaya in the northeastern area as well (Hegde and Krishnaswamy, 2021a).

Like other orchids, *Nervilia crociformis* has a specialized relationship with mycorrhizal fungi, which are essential for its germination and growth (Hegde and Krishnaswamy, 2021b). These fungi assist the orchid in obtaining nutrients and water from the soil. Sometimes pathogenic fungi also infect this species. *Colletotrichum* is one of them (Fu et al., 2019). *Colletotrichum* species are frequently discovered in a variety of plant hosts as diseases, endophytes, and infrequently saprobes (Wikee et al. 2011). It contains a number of very significant plant pathogens that infect a wide range of woody and herbaceous plants (Hyde et al. 2018). A unique fungus called *Colletotrichum fructicola* is a member of the *C. gloeosporioides* complex, a major group of plant pests and pathogens that impact several different crops (Cannon et al., 2008; Weir et al., 2012). *C. fructicola* was first discovered by Prihastuti et al. (2009) as the cause of coffee berry disease in *Coffea arabica* in northern Thailand. Later Rojas et al. (2010) identified it as a leaf endophyte in Central America. Since that time, *C. fructicola* has been linked to bitter rot, anthracnose, and leaf spot diseases in a variety of woody or herbaceous plants that thrive in temperate, subtropical, and tropical regions worldwide (Cannon et al., 2012). As this species is not common in terrestrial orchids, the characterization of *Colletotrichum* species associated with orchids in India is not yet explored.

Microscopic and molecular characterization of fungi is important to identify them. Many *Colletotrichum* species have historically been named by their hosts, this shows that different species have different host preferences (Baroncelli et al., 2018). However, the systematics of *Colletotrichum* has proven to be quite challenging because there aren't many trustworthy morphological traits, leaving species boundaries unclear (Canon et al., 2012). Phylogenetic analysis on the basis of nucleic acid sequences has been successfully developed to differentiate species from their own genera (Cai et al, 2009). In fungal molecular systematics, nuclear-encoded ribosomal RNA genes (rDNA) have been the main subject of study. For phylogenetic analyses of distantly related organisms, the coding areas of the 18S, 5.8S, and 28S nuclear rDNA genes are valuable because they are largely conserved across fungi and exhibit low sequence variation between closely related species (Crouch et al., 2009). Two internal transcribed spacers, *ITS 1* and *ITS 2*, which exhibit greater rates of divergence, lie between the conserved areas in each repeat unit (Crouch et al., 2009). These *ITS* regions have received the greatest DNA sequence coverage in fungi so far. Therefore, we recommend a polyphasic approach to recognize associated fungi with *Nervilia crociformis.* The objective of this study was polyphasic, we combined anatomical and morphological identification with molecular analysis using *ITS* for precise identification of the *Colletotrichum* species.

2. MATERIAL AND METHODS

Sample collection: *Nervilia crociformis* and rhizosphere soil samples were collected from the moist deciduous forests and from the adjoining grasslands close to the Shola forests of Kemmangundi, Western Ghats, India. The plant was carefully excavated to prevent root system injury because orchids have fragile root systems. Clean polythene bags were used to collect and label soil samples from the rhizosphere and the subterranean portion (root, tuber, and pseudobulb). Specimens of orchid plants were conserved and named using taxonomic keys found in different flora. (Abraham & Vatsala, 1981). It was done using Forrset et al., (2019) wet technique of preparing herbaria.

2.1 Root colonization studies

Three Plant roots, tubers, and pseudobulbs without visible damage were collected for root colonization studies. For colonization in the cortical cells, thin free-hand portions of the orchid plant, such as roots, tubers, and pseudobulbs, were observed under the light microscope. Tryphan blue (0.05% in lactophenol) was used to stain the plant material, which was then examined under a light microscope for signs of fungal colonization, such as hyphal coils in the cortical cells. In each part, undamaged and digested highly coiled hyphal pelotons were seen and evaluated. The method of calculation was used to determine colonization density: Percent colonization = (Number of colonized root segments X 100 /Number of total segments examined), as used by Manoharachary and Tilak (2015).

2.2 Fungal isolation:

By plating pieces of surface-sterilized roots or tubers on the appropriate media, mycorrhizal fungi were isolated according to standard protocols. Roots were cut into 5–10 mm pieces, aseptically plated on Potato Dextrose Agar (PDA) medium with streptomycin (50 g/mL), and then let to set for an incubation period of 25^{o}C. To create the pure culture, fungi grew from the cut ends. Prior to trying to identify them, these isolates were further cultured on PDA at 25^{o}C for 4 weeks under regular diurnal circumstances. This allowed sclerotia and chlamydospores to develop and mature (Ma et al., 2018).

2.3. Fungal isolate purification and maintenance:

The hyphal tip technique was used to purify the fungal isolates. They were kept in the refrigerator on PDA slants for future research. This method was utilized for fungi like Rhizoctonia, which do not sporulate in culture, or species that commonly create defective cultural variations from germinated conidia (Zhu et al., 2008)**.**

2.4 Transmission electron microscopic (TEM) identification of the fungus:

Root samples were fixed at 4 degrees Celsius in modified Karnovsky's fluid buffered with 0.1 M sodium phosphate buffer. After 16 hours, the roots were thoroughly washed with buffer. The samples were also dehydrated in graded acetone solutions after being post-fixed for 2 hours at 4 degrees Celsius in 1% osmium tetroxide. The samples were cut into ultrathin slices with an Ultracut E (Reichert Jung) ultramicrotome after being embedded in CY 212 Araldite. The slices were stained in lead citrate and alcoholic uranyl acetate for ten minutes each before

being studied with a Philips CM-10 transmission electron microscope.

2.5 Molecular characterization of fungus:

The pure cultures (6 samples) of the mycorrhizal fungi obtained from the tuber/root of terrestrial orchids were used for molecular analysis.

2.5.1 DNA isolation: Genomic DNA was extracted from the mycelial using the CTAB method with slight modifications. Polymerized chain reaction (PCR) was performed in a Thermocycler (PTC- 100TM programmable thermal controller, USA) to produce multiple copies of a specified DNA. Universal forward and reverse primers for fungal *ITS*(Srivastava and Manjunath, 2020). These primers were purchased from Chromous Biotech Pvt. Ltd. Bangalore, India. 50 μL of PCR reaction mixtures were used to amplify the *ITS* regions. The amplification of the *ITS* regions of the fungal DNA was performed using MyGene^{TM} Thermal Cycler-MG96G with Standard PCR conditions.

2.5.2 Sequence analysis: DNA was sequenced by the chain termination method using an ABI 3130 Genetic Analyzer. MEGA software, version 6.0, was used for phylogenetic analysis and ClustalW for sequence alignments. Utilizing the neighbor-joining (NJ) technique, bootstrapping of 1000 and the p-distance substitution model were used to construct a phylogenetic tree (Tamura et al., 2013).

3. RESULTS AND DISCUSSION:

3.1 Root colonization studies

Nervilia crociformis with very thin roots, pseudobulb and tubers were selected for the study (Figure 1). Microscopic studies of roots and tuber sections were conducted using light microscopy. It showed hyphal colonization on the surface of roots (Figure 2a) as well as in the inter and intra-cellular spaces of the root cortex (Figure 2b). Extensive inter and intracellular root colonization with intact and digested pelotons was also noted (Figure 2c, d). Pelotons were observed a thin-walled hyphae lumps inside the cortical cells of the root. Tan et al., (2014) also identified as fungal colony of *Colletotrichum fructicola* based on morphological characteristics and found similar pelotons.

3.1 TEM studies of roots

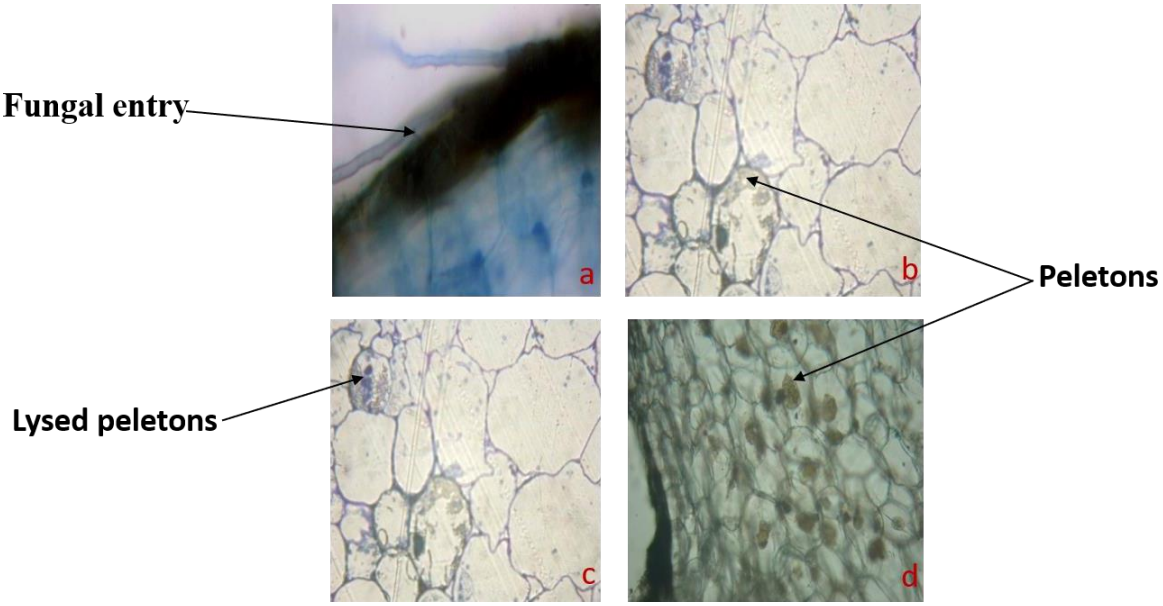
TEM studies showed that the rhizodermis was uniseriate, and the cortex was thin-walled (6- to 8-cell broad) and parenchymatous. Both intact and digested pelotons were seen in the inner cortex, which reached up to 2-3 layers. Up to 40% of an area was colonized. The rhizodermis, endodermis, and vascular area lacked pelotons. Few pelotons were visible in the peripheral cells in the tuber's transverse slice Similar pelotons structure of *Colletotrichum fructicola* was seen by Jiang et al (2014) in young pear fruit causing black spots.

3.2 Isolation of fungal hyphae

Fungi were isolated from the roots, tubers, and pseudobulbs of *Nervilia crociformis* (Figures 3a and 3b). Pure cultures were best grown on a PDA medium. Colonies on PDA at first white, become grey to dark grey at the center with age, in reverse greyish green with white became black with age. The maximum size attained by colonies was 8.3cm in diameter in 7 days. The light microscopic view showed aerial mycelium was pale grey, dense, and cottony and without visible conidial masses (Figure 3c).

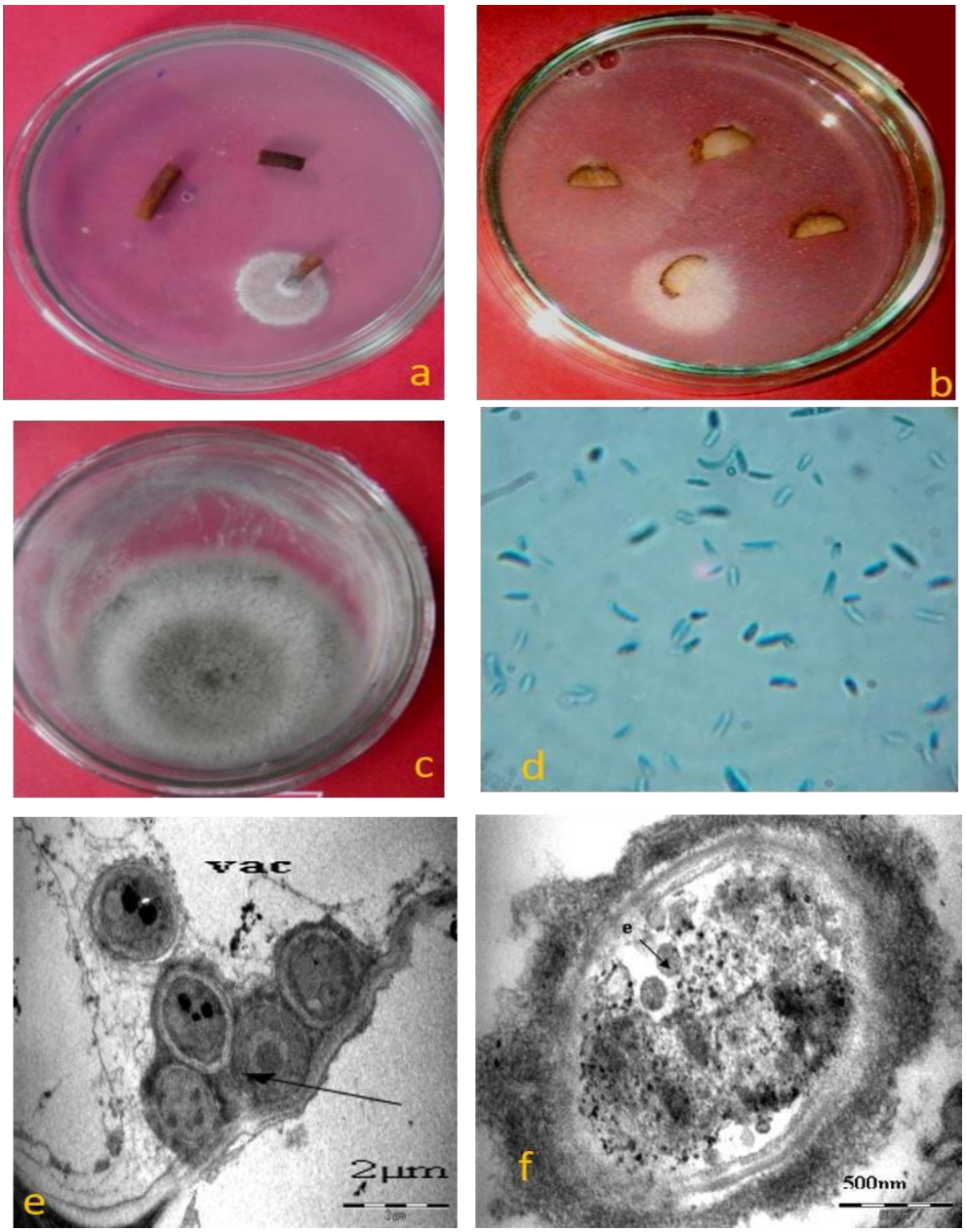
Figure 1: Tuber of *Nervilia crociformis*

Figure 2: Anatomical studies in *Nervilia crociformis*



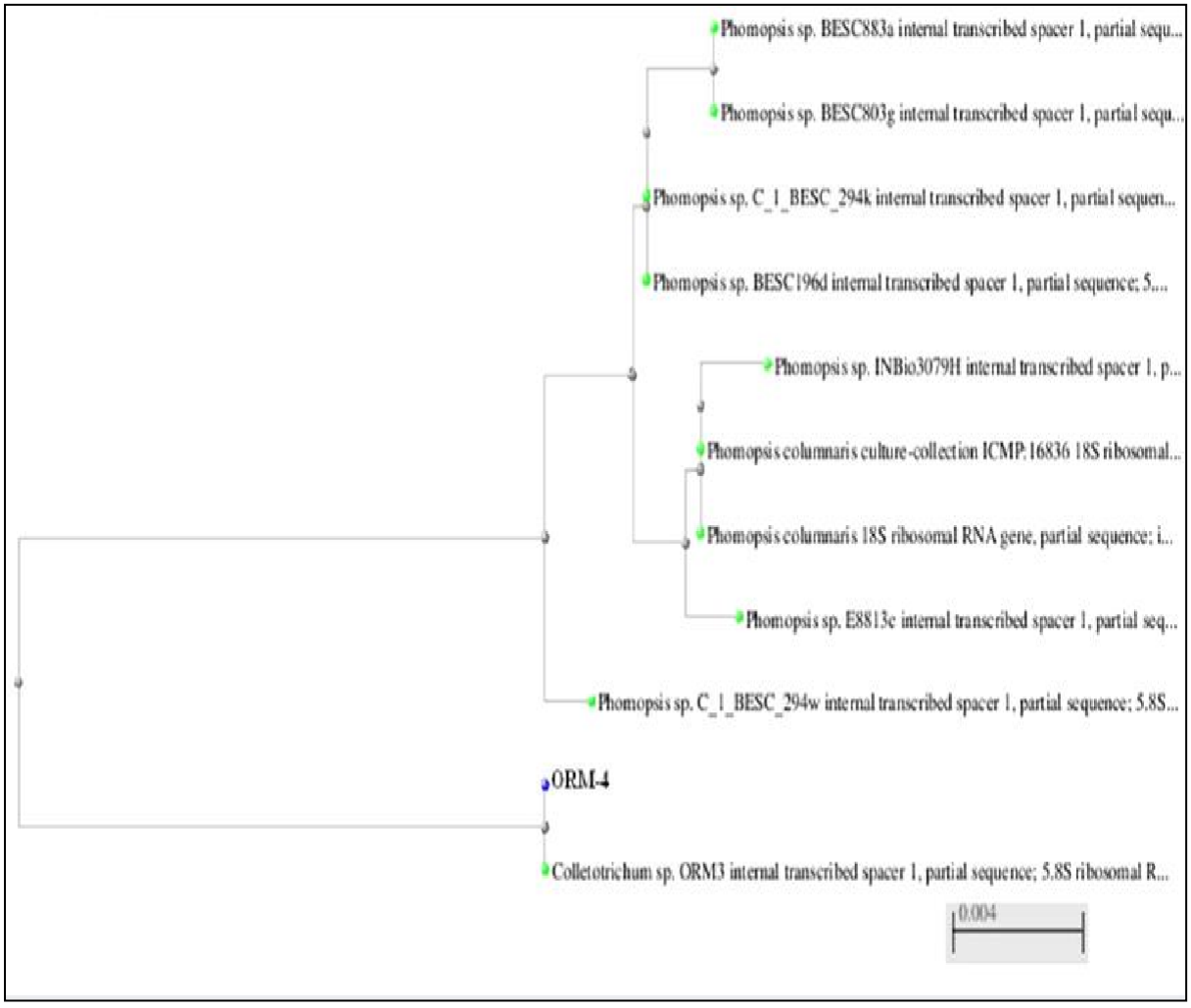
a. Fungal entry into the root, b. Compact peletons in root cortex
c. Lysed peletons in the root; d. Intact peletons in rhizome

Figure 3: Isolation of fungus in *Nervilia crociformis*



a. Incubation of root; b. Incubation of rhizome; c. Fungal isolate; d. Transverse electron microscopic view of *Colletotrichum fruticola*; e. Fungal hyphae in host cell; f. Structure of hyphae in the host cytoplasm

Figure 4: Phylogenetic analysis of *Colletotrichum fructicola*



3.2 Anatomical characterization of fungal hyphae

TEM characterization of fungi showed the hyphae are thinly enveloped by host cytoplasm (Figure 3d). Hyphae were noted as obtuse, somewhat rounded, occasionally oblong cells, and cylindrical (Figure 3e). The host cytoplasm could be sparsely encasing the hyphae (Figure 3f). The inner layer of the internal hyphae was typically 60 nm thick, whereas the outer layer was more granular and 100–200 nm thick. Sclerotia were absent and single-celled, smooth- walled conidia of size 9.5-14 x 3-4.5µm were present.

3.3 Molecular characterization of fungal hyphae

As morphological and anatomical characterization results could be confirmed only after phylogenetic analysis. Molecular marker approaches have increased the precision and speed of identifying and classifying phytopathogenic fungi (Srivastava and Manjunath, 2020). DNA was extracted and purified by the standard methods described. The amplification success of

loci, namely, *ITS* was 98%. *ITS* sequence data of the fungal isolate from the tuber of *Nervilia crociformis* showed a close (98%) resemblance to many *Colletotrichum* species on NCBI-BLAST (GenBank Accession No. KC920479). NJ- based tree of *ITS* sequences showed higher bootstrap values sample (ORM- 4) and was clustered with other species of genus *Colletotrichum*. The phylogenetic tree of the fungal isolate is given (Fig. 4). Fu et al., (2019) and Baroncelli et al., (2018) also identified a few species of *Colletotrichum* using similar methods.

CONCLUSION: Anatomical and molecular characterization of a fungal isolate of *Nervilia crociformis* orchid roots confirmed the presence of *Colletotrichum fructicola.* This fungus may form a good mycorrhizal association with different terrestrial orchid plants in India so further verification is needed.

CONFLICT OF INTEREST: None

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