Original Research Article

Morphological and molecular characterization of endophytic fungi associated with Cocoa (*Theobroma cacao* L.) in India

ABSTRACT

Aim: To identify cocoa associated endophytic fungi through morphological and molecular techniques.

Place and duration of study: Department of Plant Pathology, TNAU, Coimbatore, Tamil Nadu from April 2018 to November 2019.

Methodology: Endophytic fungal isolates were isolated from different parts of cocoa using Petrini method. Isolated endophytic fungal strains were grown in Malt extract broth and total genomic DNA was isolated and amplified using universal primers ITS1F and ITS4R. Amplified rDNA was visualized and documented.

Results: Morphological and molecular characterization of six endophytic fungi revealed that they are from four different taxa *viz.*, *Lasiodiplodia pseudotheobromae* PAK-7, *Arthrinium rasikravindrae* P11, *Arthrinium rasikravindrae* P12, *Diaporthe* sp. Vef-3, *Lasiodiplodia theobromae* TN-R-3, *Colletotrichum* sp. TN-9-2 belonging to four different families *viz.,* Botryosphaeriaceae, Apiosporaceae, Diaporthaceae and Glomorellaceae under Phylum Ascomycota.

Conclusion: The present study indicates the distribution and diversity of fungal endophytes in different plant parts of the cocoa tree in south India.

Keywords: *cocoa, endophytic fungi, ITS.*

1.Introduction:

Endophytes are a diverse group of microbes living inside plants without any external symptoms. They influence plant health by the production of phytohormones and antimicrobial compounds. They also compete with plant pathogens for ecological niche and nutrients. The major group of endophytic fungi belongs to Ascomycetes and anamorphic fungi [1]. Basidiomycetes and zygomycetes are rarely encountered. These fungi have been identified in nearly 3,00,000 plant species and they dwell in all plant parts (leaf, flower, petiole, root, fruit) [23]. There existed a co-evolution between endophyte and its host which lead to low virulence [17]. The interaction between endophyte and the host ranges from symbiotic, mutualistic to pathogenic [21, 20] depending on the natural environmental condition and plant health [15]. Cocoa (*Theobroma cacao* L.) is an introduced tropical rainforest crop grown as an intercrop or mixed crop within a coconut, arecanut or oil palm plantations in India. In India Cocoa is being cultivated in the States of Kerala, Karnataka, Andhra Pradesh and Tamil Nadu in an area of 78,000 ha with a total production of 16,050 MT. The average productivity of cocoa in Indian is 475 kg/ha (www.dccd.gov.in). The present study was aimed to identify the endophytic fungi of cocoa both from morphologically and molecularly using PCR-based molecular techniques which helps to know endophytic fungal diversity in the healthy cocoa tree.

2. Materials and method

2.1 Sample collection

During September 2017 asymptomatic plant from a disease-prone area was located and marked. Different plant parts were excised with sterile knife and samples were carried to the lab in a zip-lock cover and kept at 4℃ until further use (Table 1).

Table 1. Geographical distribution and isolation sources of endophytic fungi.

Sl.no	Isolate	Intercropping system	Place of collection	Plant part
1.	Pak-7	Coconut and cocoa	Palakad, Kerala	Petiole
2.	P11	Coconut and cocoa	Palakad, Kerala	Petiole
3.	P12	Coconut and cocoa	Palakad, Kerala	Petiole
4.	VEF-3	Arecanut and cocoa	CPCRI,RS, Vittal, Karnataka	Petiole
5.	TN-R-3	Coconut and cocoa	TNAU, Coimbatore, TN	Root
6.	TN-9-2	Coconut and cocoa	TNAU, Coimbatore, TN	Root

2.2 Isolation of endophytic fungi

Isolation of endophytic fungi from cocoa was done by following Petrini method [11]. Sterilization involved washing of collected plant samples in running tap water followed by drying. Washed plant parts *viz*, stem, leaf, petiole and roots were excised into small bits with sterile scalpel and surface sterilized with 4% NaOCl for 30sec-1min followed by two times rinse in sterile distilled water. A second wash was given with 70% ethanol for 1-2 min. the surface sterilization efficiency was checked by plating last wash water on malt extract agar which served as a control. Plant parts were properly dried before placing on MEA and incubated at 27±2℃ for 7-14 days depending on the growth of the endophyte.

2.3 Morphological characterization

Initial identification of fungal endophytes was done based on morphological observations. A loop full of mycelium was taken on a cavity slide and observed under the Stereo Binocular Phase-contrast microscope (LEICA DM2000 LED) for the presence of structures like mycelium (color & septation) sexual and asexual structures/spores. Images were captured and analyzed using an image analyzer.

2.4 ITS-PCR and sequencing

Molecular characterization was done following ITS-PCR and sequencing of the amplified DNA fragment. Cultures were grown in MEB ((Malt extract broth) for 7 days at 27±2℃ inside a BOD incubator. Approximately 200mg of freeze-dried (-20℃) mycelium was macerated with 1-2ml of CTAB buffer (10%CTAB- 10ml, 1M Tris base-5ml, 5M EDTA-2ml (pH-8), mercaptoethanol-1ml and distilled water-18ml) warmed at 60℃. Transferred 700µl of macerate into a eppendorf tube (2ml) and warmed at 65℃ for 10min. Added 750 µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) and inverted it to form an emulsion. Centrifuged the content @10000rpm for 10 min. The supernatant was transferred into a sterile eppendorf tube and 0.5vol of 5M NaCl (150µl) and 2vol (600µl) of ice-cold Isopropanol was added and incubated @ -20℃ for 12h/overnight. Later tubes were inverted 2-3 times and centrifuged @13000rpm at 4℃ for 10min. supernatant was discarded and the pellet was washed with 70% ethanol. After proper drying pellet was suspended in 50µL of TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA). The quantity of DNA was checked by electrophoresis in 0.8 % agarose (HiMedia Pvt Ltd. India) gel supplemented with 2µl of ethidium bromide for 45min at 90V in 1X TAE buffer (Tris base -4.84g, acetic acid-1.09ml, EDTA-0.292g, distilled water-100ml, pH-8.1—8.2) and visualized under UV transilluminator.

Before PCR the concentration of genomic DNA was adjusted to 200ng. Later ITS-PCR was carried out using internal transcribed spacer primers ITS1F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4R

(5'-TCCTCCGCTTATTGATATGC-3') with a final reaction mixture concentration of 55µl (smart primer - 20 µl, ITS1F- 4 µl, ITS4R- 4 µl, d_2H_20- 8 µl and DNA-8 µl). Amplification was carried out in Eppendorf thermal cycler with an initial denaturation at 95℃ for 3 min, denaturation at 95℃ for 1min, annealing at 55℃ for 1 min, extension at 72℃ for 1 min followed by a final extension at 72℃ for 5 min with 35 cycles. The PCR amplified products were visualized in 1.2% agarose gel and documented. The amplified product was sent to AgriGenome Lab Pvt Ltd for purification and sequencing.

3. Results and Discussion

The current study was carried out to find the associated endophytic fungi in healthy cocoa trees in a disease-prone area. Isolated endophytic fungi were morphologically and molecularly characterized following microscopic observations and rDNA sequence blast in NCBI database USA.

3.1 Morphological characterization

Morphological characterization is one of the oldest and reliable methods for the identification of any microorganisms. In the present investigation, cocoa endophytic fungi were characterized morphologically by studying mycelial and spore characters. Isolate PAK-7 (*Lasiodiplodia pseudotheobromae)* and TN-R-3 (*Lasiodiplodia theobromae*) produced colored and septate mycelium without any sexual or asexual structures. Similarly, morphological characterization of endophytic and saprobic isolates of *Lasiodiplodia pseudotheobromae* NI173 was studied where it produced brown colored conidia with 19–25×12–15µm size [3].

The genus *Arthrinium* is widespread and occurs as a saprobe on a different range of substrate [1]. It is a plant pathogen reported to cause diseases like kernel blight of barley [9], damping-off of wheat [10]. Its endophytic nature has been reported in lichens, marine algae plant tissues [12,19,7]. Hanada et al. [6] reported the endophytic nature of *Arthrinium sp* in a cocoa tree. In the present study *Arthrinium rasikravindrae* isolates P11 and *Arthrinium rasikravindrae* P12 produced olive green colored lenticular single-celled conidia with septate olive green colored mycelium. The present finding is in agreement with Singh et al. [18] where he isolated *Arthrinium rasikravindrii* sp. nov. from the soil and it produced dark brown lenticular conidia with hyaline equatorial germ slits together with balloon-shaped, anomalous conidia.

The Isolate TN-9-2 (*Colletotrichum* sp.) produced hyaline dumble shaped single-celled conidia this is in agreement with Rojas et al. [13]. The endophytic nature of *Diaporthe* sp., *Colletotrichum* sp and *Lasiodiplodia* sp in cocoa was reported by Rubini et al. [14]. Ding et al.[4] studied the morphological characters of endophytic *Diaporthe* sp. isolated from *Camptotheca acuminate* from China. It produced a gray-colored colony with branched and septate mycelium. This finding matching with that of the present study where isolate VEF-3 (*Diaporthe* sp.) produced light brown to dark-colored septate mycelium with few alpha conidia in MEA medium.

3.2 Molecular characterization and phylogeny

3.2.1 ITS-PCR and sequencing

Isolated and amplified rDNA from endophytic fungi were subjected to PCR analysis. The DNA band size ranged from 336-457bp (Fig 2). ITS rDNA sequence analysis of PAK-7 and TN-R-3 showed cent percent similarity with *Lasiodiplodia pseudotheobromae* and *Lasiodiplodia theobromae* respectively (Table 2). Similarly de Silva et al.[3] did molecular characterization of *Lasiodiplodia pseudotheobromae* isolated from Magnolia forest plants using combined ITS, tef1 and tub2 genes.

The endophytic *C. gloeosporioides* strains from asymptomatic leaves, anthracnose lesions on leaves and fruits of *Theobroma cacao* (cacao) were subjected to multilocus phylogeny to distinguish host-associated pathogens from asymptomatic cocoa plant parts [13]. Endophytic Isolate TN-9-2 from the present investigation produced dumble shaped hyaline conidia which is the characteristic feature of genera *Colletotrichum.* The sequencing result showed that it matched with that of *Colletotrichum* sp. voucher UOM 1290T with 100% identity (Table 2). Lima et al.[8] identified *C. gloeosporioides*, *C. boninense*, and *C. simmondsii* in Brazilian pepper tree using combined morphological and PCR

taxon-specific primer with CaInt/ITS4, CgInt/ITS4, and Col1/ITS4, which amplify specific bands in *C. acutatum, C. gloeosporioides lato sensu, and Colletotrichum boninensis*, respectively.

Hanada et al.[6] reported the endophytic nature of *Arthrinium sp* in a cocoa tree. In the present study endophytic isolates, P11 and P12 were isolated from cocoa and they were molecularly characterized using ITS1F and ITS4R primers. The sequence result showed 100% identity with *Arthrinium rasikavindii* (Table 2). Ghasemi et al. [5] identified endophytic *Arthrinium arundinis* from oak trees in Arasbaran forest based on morphological characters and ITS-rDNA and Tub region sequence data.

Many reports are suggesting the endophytic nature of *Diaporthe* sp. In cocoa as well as in the forest tree. In the present study endophytic fungal isolate Vef-3 sequence data matched with taxa *Diaporthe* sp. with 100% similarity.

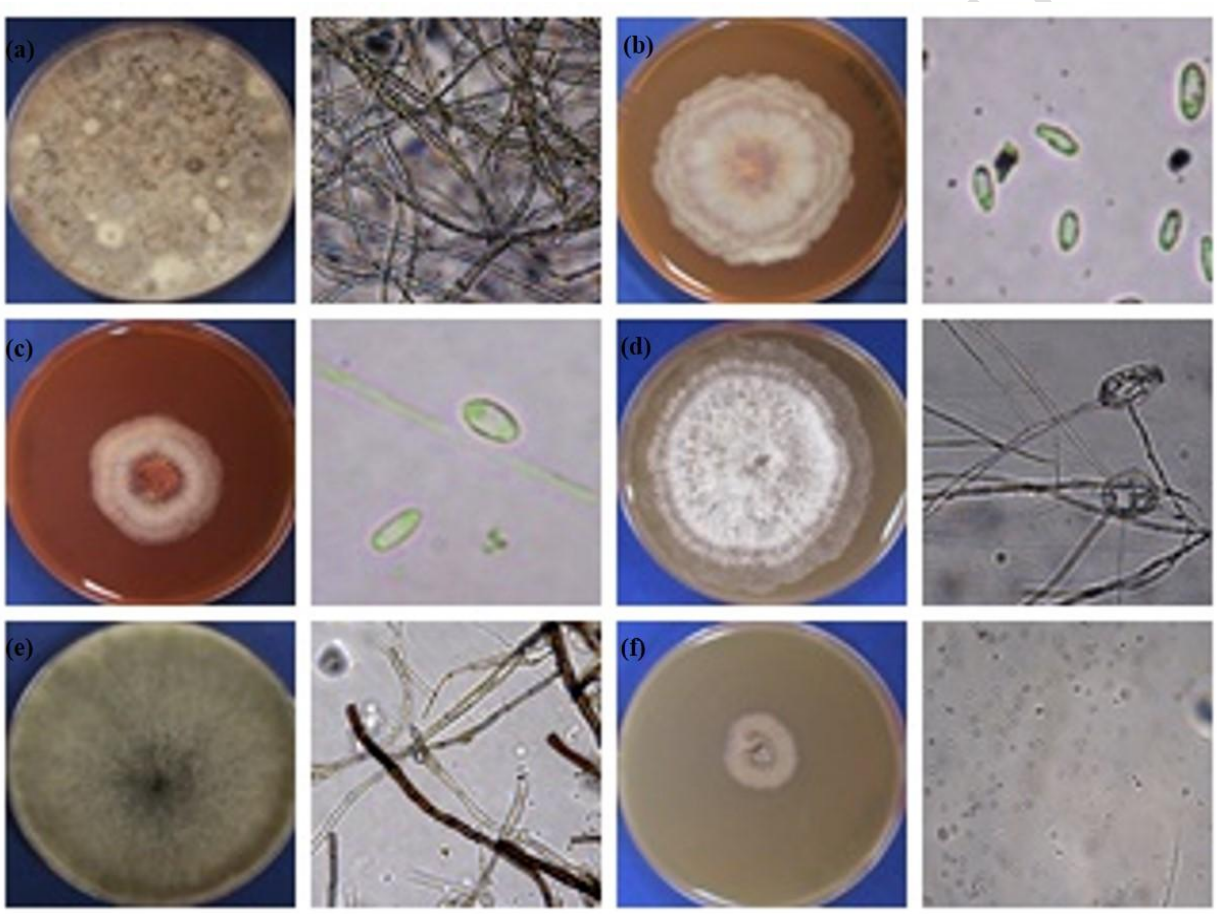

Fig 1. Macroscopic and microscopic observations on endophytic fungal isolates of cocoa. a-PAK-7 (*Lasiodiplodia pseudotheobromae*), b- c *Arthrinium rasikravindrae* P11 and P12 isolates conidia, d-Vef-3 (*Diaporthe* sp.) melanized mycelium, e- TN-R-3 (*Lasiodiplodia theobromae*) melanized mycelium, f- TN-9-2 (*Colletotrichum* sp.) conidia.

Table 1. A comparison on morphological characters of different endophytic fungal isolates of cocoa.

Sl. no	Isolate	Organism	Culture color	Margin	Mycelium	Spore
1	PAK-7	*Lasiodiplodia pseudotheobromae*	Lichen Green	Entire	Light brown color	Sterile
2	P11	*Arthrinium rasikravindrae*	Pinkish white	Irregular/Wavy	Olive green color	Olive green color conidia
3	P12	*Arthrinium*	Pinkish	Fringed	Olive green	Olive green

		rasikravindrae	white		color	color conidia
4	Vef-3	*Diaporthe* sp.	Chalk white	Fringed	Light brown to dark brown	Colorless Alpha conidia
5	TN-R-3	*Lasiodiplodia theobromae*	Ash gray	Irregular	Chocolate brown and septate	Sterile
6	TN-9-2	*Colletotrichum* sp.	Ash gray	Irregular	Hyaline	Dumble shaped single-celled hyaline conidia

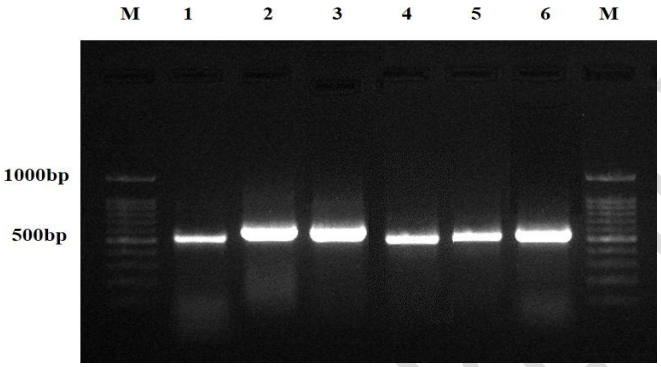

Fig 2. Molecular characterization of the endophytic fungi of cocoa using ITS1F and ITS4R primers: M-1.5kb Marker, 1- PAK-7 (*Lasiodiplodia pseudotheobromae*), 2- P11 (*Arthrinium rasikravindrae*), 3-P12 (*Arthrinium rasikravindrae*), 4- Vef-3 (*Diaporthe* sp.), 5 -TN-R-3 (*Lasiodiplodia theobromae*), 6 -TN-9-2 (*Colletotrichum* sp.).

Table 2. Molecular characterization of endophytic fungal isolates of cocoa, based on NCBI sequence blast result.

Sl. no	Isolate	Accession number	Closest match	Query coverage (%)	Percent identity
1.	Pak-7	MN418007	*Lasiodiplodia pseudotheobromae*	100	100
2.	P11	MN414159	*Arthrinium rasikravindrae*	100	100
3.	P12	MN413149	*Arthrinium rasikravindrae*	100	100
4.	VEF-3	MN420881	*Diaporthe* sp.	100	100
5.	TN-R-3	MN400974	*Lasiodiplodia theobromae*	100	100
6.	TN-9-2	MN412519	*Colletotrichum* sp	100	100

3.2.2 Phylogenetic analysis

The phylogenetic tree is used to represent evolutionary relationships between organisms which are having ancestors in common. In the present study evolutionary tree was constructed using MEGA7 software with four clades representing four different genera *Lasiodiplodia, Diaporthe, Arthrinium,* and *Colletotrichum* belonging to the same Phylum Ascomycota representing four different families (Fig 3). The genus *Lasiodiodiplodia* was from Botryosphaeriaceae, *Arthrinium* from Apiosporaceae, *Colletotrichum* from Glomerellaceae and *Diaporthe* from Diaporthacea. The first clade consisted of two sequences of *Lasiodiodiplodia* which clustered with twelve sequences from NCBI database

(MN046825, MN173966, MK808536, MK883476, MN398978, MN256461, MK808133, MH731278, KY969640, KF913502, KF164311, JX868715) with 95% bootstrap value. The second clade comprised of the genus Arthrinium, which consisted of two sequences of endophytic fungi of cocoa which clustered with five NCBI database sequences (MK304236, KT722600, MK014896, MH498538o, and MK850243) with 88 % bootstrap value. The third clade comprised of the *Colletotrichum* genus which consisted of one sequence of endophytic fungi that clustered with four sequences from the NCBI database (MK914629, MH883641, MH388336 and MK300802) with 90% bootstrap value. The fourth clade comprised the genus *Diaporthe* with one endophytic fungal sequence which clustered with four NCBI database sequences (KF435362, KF688124, MK335817 and KU712435) with bootstrap value 65%.

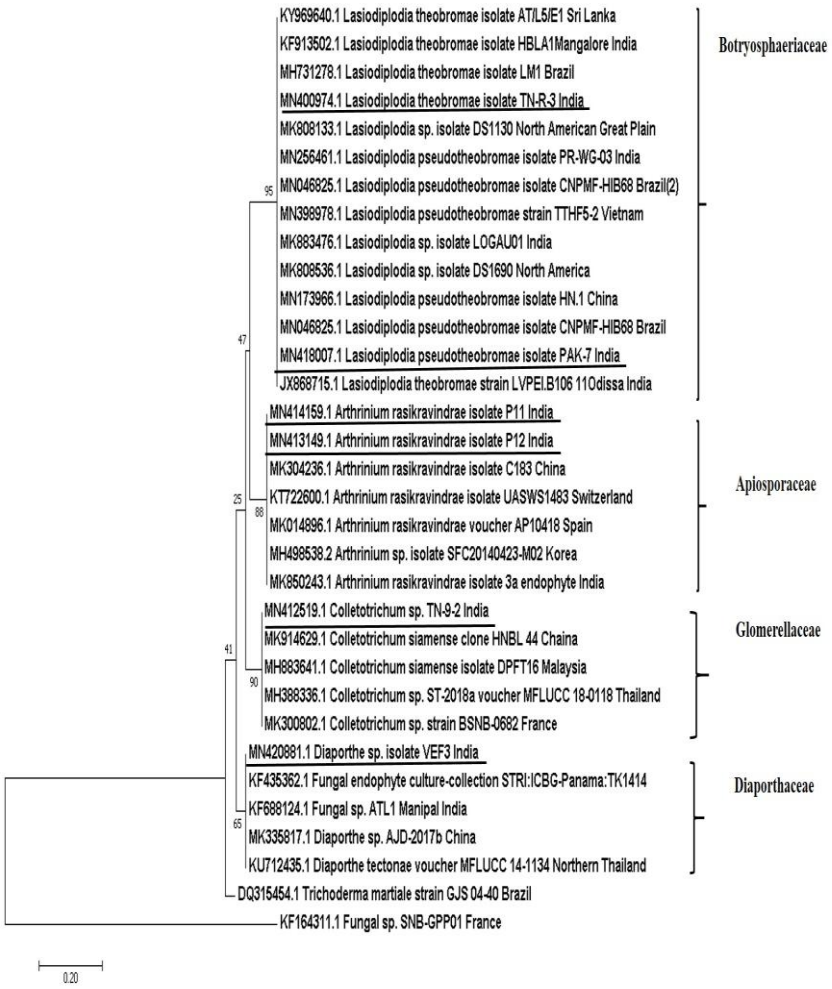

Fig 3. Phylogenetic relationship between six endophytic fungal isolates of *Theobroma cacao* based on ITS rDNA sequence by Neighbour-joining (NJ) method following the bootstrap test (1000 replication).The evolutionary distances were computed using the Maximum Composite Likelihood method following Gamma distribution using MEGA7 Software.

In the present investigation, all six isolated endophytic fungal taxa belonged to the phylum Ascomycota. This is in agreement with the findings of Evans et al.[8] and Crozier et al.[3] where they isolated and characterized fungal community inhabiting in root, stem and branches of cocoa. The studies on the fungal endophytic community in Cocoa are not well known so efforts may be taken to characterize these microbes to characterize it both morphologically and molecularly.

4. Conclusion

Studies on endophytes are a new field of research that gives us a way to understand the diversity of the endophytic community in a plant. The present study aims to identify endophytic fungal isolates of cocoa based on morphological and molecular characters. A total of six endophytic fungal isolates were studied and they are all from the same phylum Ascomycota, belonging to different families. Isolates *Lasiodiplodia pseudotheobromae* PAK-7 and *Lasiodiplodia theobromae* TN-R-3 are from Botryosphaeriaceae, *Arthrinium rasikravindrae* P11 and *Arthrinium rasikravindrae* P12 were from Apiosporaceae, *Diaporthe* sp Vef-3 was from Diaporthaceae and *Colletotrichum* sp. TN-9-2 was from Glomorellaceae which indicated the fungal diversity in different plant parts of cocoa. For better understanding further studies may be carried out to confirm the endophytic nature of asymptomatic fungal pathogens as well as antagonistic microbes in a cocoa tree.

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