RESEARCH ARTICLE

Molecular phylogeny-based identification of *Colletotrichum endophytica* and *C. siamense* as causal agents of avocado anthracnose in Sri Lanka

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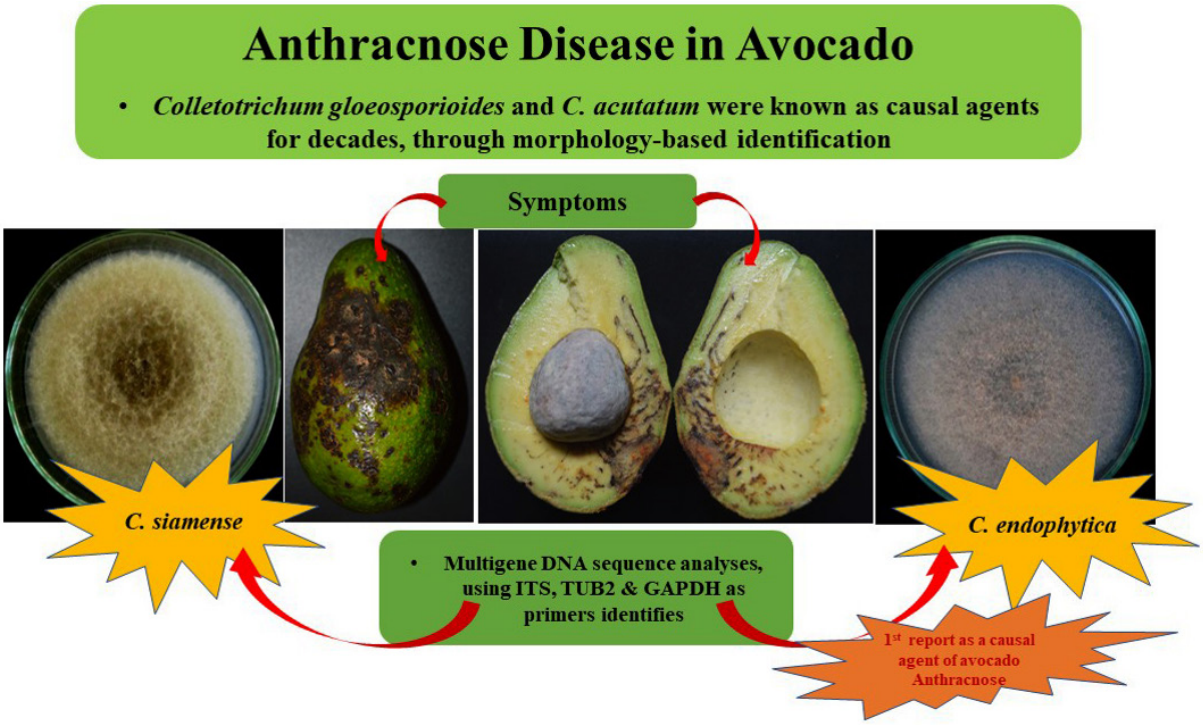

Highlights

- Anthracnose is a most destructive disease in avocado causing significant postharvest losses.
- *Colletotrichum* isolates from avocado anthracnose were characterized by multigene DNA sequence analyses.
- *Colletotrichum endophytica* & *C. siamense* were identified as causing avocado anthracnose in Sri Lanka.
- Molecular differences between the two species did not correlate with morphological differences.
- This is the first report of *Colletotrichum endophytica* as a causal agent of avocado anthracnose.

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Molecular phylogeny-based identification of *Colletotrichum endophytica* and *C. siamense* as causal agents of avocado anthracnose in Sri Lanka

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Abstract: Avocado (*Persea americana*) is a sub-tropical fruit with high nutritional value and numerous health benefits. Among the postharvest fungal diseases that affect ripe avocados, anthracnose is one of the most destructive disease worldwide, causing significant postharvest fruit losses and limiting shelf life. Over 15 *Colletotrichum* species have been reported as causing avocado anthracnose from avocado growing countries in the world. In the present study, 35 *Colletotrichum* isolates were obtained from ripe avocados showing anthracnose symptoms, collected from the Central and North Western Province of Sri Lanka. Fifteen randomly selected isolates were subjected to DNA sequence analysis using ITS, TUB2, and GAPDH regions. Species affiliations and identities of the resulting sequences were determined through similarity-based searches of the NCBI GenBank Database. Based on the combined phylogentic analysis of three gene regions, nine and six isolates were identified as *C. endophytica* and *C. siamense* respectively, both belonging to the *C. gloeosporioides* species complex. Of the two species, *C. endophytica* is reported as a causal agent of avocado anthracnose for the first time.

Keywords: Postharvest loss; β-tubulin 2; GAPDH; *Colletotrichum gloeosporioides* species complex.

INTRODUCTION

Avocado (*Persea americana* Mill, Lauraceae) is native to Central America and southern Mexico and believed to have originated about 12,000 years ago, based on archeological evidence. The avocado is botanically classified in to three races, West Indian (WI), Mexico (XX), and Gautemalan (G). Systematic studies have classified more than 500 cultivars worldwide and there is a great variability in fruit traits not only between races but also among cultivars within races. The peel of some cultivars (*e.g.* Hass) changes from green to black or purple. The pericarp, which is the fruit tissue proper excluding the seed, comprises the rind (exocarp), the fleshy edible portion (mesocarp), and a thin layer next to the seed coat (endocarp) (Biale and Young, 1971).

Avocado is a fruit with high nutritional value and numerous beneficial health effects (Meyer and Terry, 2010). The fruit is a rich source of fats, particularly of monounsaturated fatty acids. The most abundant fatty acid is oleic acid that is known to reduce inflammation, a risk factor for cardiovascular diseases, and beneficial effects on cancer (Yoneyama *et al*., 2007). Health benefits of avocados are due to the presence of numerous bioactive phytochemicals (Adikaram *et al*., 1992; Tabeshpour *et al*., 2017). The fruit contains rare sugars of high carbon number and is relatively rich in certain vitamins, dietary fibre, and minerals. The fruit has high oil content and low sugar, hence recommended as a high energy food for people with diabetics. Avocado is a climacteric fruit, with a marked rise in respiration rate, followed by a decline.

Genus *Colletotrichum* is composed of plant pathogens of worldwide importance, particularly causing anthracnose in several tropical fruit species. Anthracnose disease (Sivanathan and Adikaram, 1989), caused by *Colletotrichum* species, and the stem-end rot (Madhupani and Adikaram, 2017) incited by several fungal pathogens, including *Lasiodiplodia theobromae*, are major constraints to the avocado industry, causing heavy fruit losses after harvest and limiting their marketing potential and shelf-life.

Anthracnose disease was believed to be caused by *Colletotrichum gloeosporioides* (Sivanathan and Adikaram, 1989) and *C. acutatum* (Hartill, 1991) for decades in the 19th century. More recent molecular studies, have revealed the association of over 15 *Colletotrichum* species with the anthracnose disease from both avocado growing and marketing countries of the world. Among them, the most significant number of species recorded, from a single country, was Israel where multi-locus phylogenetic analyses using ITS, act, ApMat, cal, chs1, GAPDH, GS, HIS3, TUB2 gene/ markers, identified eight previously described species, *C. aenigma*, *C. alienum*, *C. fructicola*, *C. gloeosporioides sensu stricto*, *C. karstii*, *C. nupharicola*, *C. siamense*, *C. theobromicola*, and a novel species, *C. perseae*, as causing avocado anthracnose, confirming their pathogenicity (Sharma *et al*., 2017).

Talhinhas *et al*. (2002) were the first to carry out multilocus-based phylogenetic analysis for *Colletotrichum* species. Using multiple sequence alignment, past phylogenetic analyses have revealed that the genus *Colletotrichum* comprises of eleven species complexes

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and 23 singletons where the *C. gloeosporioides* species complex is collective of *C. gloeosporioides s.s* and 51 closely related species (Weir *et al.,* 2012; Jayawardena *et al*., 2020). Similarly, *C. acutatum* is now considered a species complex consisting of 41 species that include *C. acutatum s.s* and its close relatives (Jayawardena *et al*., 2020).

The present study re-evaluated the *Colletotrichum* species associated with avocado anthracnose in Sri Lanka by multigene DNA sequence approach, using 35 isolates from diseased fruits collected in two major avocado producing provinces and also the semi-systemic nature of internal symptom development.

MATERIALS AND METHODS

Isolation of *Colletotrichum*

Ripe avocado fruits showing characteristic symptoms of anthracnose disease were collected from wholesale fruit stores or retail outlets in two main avocado-producing and distributing areas, Kandy (Central Province), and Kurunegala (North-Western Province) Districts, of Sri Lanka, over two fruit seasons in 2015 - 2016. Diseased fruits were brought in sealed polythene bags to the Plant Pathology laboratory at the Department of Botany, University of Peradeniya, Sri Lanka.

Colletotrichum was isolated from anthracnose lesions on 35 infected avocado fruits. Segments (5 × 5 mm^2) of infected tissues, cut from the advancing margin of anthracnose lesions in the fruit peel, were surface sterilized in 1% sodium hypochlorite (Clorox, USA) for 1 - 3 min followed by rinsing twice in sterile distilled water (SDW). The excess liquid in tissue segments was removed by placing them on sterile filter papers. Tissue pieces (4 per plate) were aseptically transferred onto PDA medium, supplemented with 50 µg mL^{-1} tetracycline to suppress bacterial growth. The plates were incubated at 28 ℃ for 5 - 7 days. The 35 isolates obtained were sub-cultured by transferring discs (6 mm diameter) of mycelium onto fresh PDA plates and allowed to grow at 28 ℃ for 14 days.

Preparation of mono-conidial cultures

A suspension of conidia of each isolate was prepared by suspending the mycelium scraped from 10 - day old cultures in sterile distilled water (SDW) and filtering through sterile glass wool. A loop-full of each suspension was streaked over thin tap water agar plates. After incubation the plates for 18 h at 28 ℃, a small piece of agar with a single germinated conidium, located by moving the objective lens (× 25) of a light microscope (Olympus CX 22) along the streak line, was cut and transferred onto fresh PDA. The plates were incubated for seven days. Pure cultures were maintained in microcentrifuge tubes (1.5 mL) containing 800 µL sterile PDA at 15 ℃ (Prihastuti *et al*., 2009) to be used in subsequent studies.

DNA extraction, PCR amplification and sequencing

Fifteen isolates, selected randomly from the initial 35 isolates, were used for molecular studies. DNA was extracted using the protocol described by Živković *et al.* (2010). Aerial mycelium (0.5 g), scraped from seven days old cultures, using a sterile inoculation loop, was placed in a sterile microcentrifuge tube (1.5 mL) containing 300 µL of extraction buffer (0.2 M Tris-HCl, 0.25 M NaCl, 25 mM EDTA, and 2% SDS, pH 8.5) and crushed well. Uncapped tubes were then placed in a boiling water bath for 5 min and allowed to cool to 25 ℃. Aliquots (200 µL) of phenol, equilibrated with the extraction buffer (vol/vol), and chloroform (200 µL) were added. The tubes were vortexed for 2 - 3 min and centrifuged at 7,647 g for 5 min. The supernatant was transferred into a new 1.5 mL microcentrifuge tube containing 200 µL of chloroform and vortexed for 30 s followed by centrifugation at 7,647 g for 15 min. The supernatant was pipetted out into a new 1.5 ml tube and 200 µL of ice-cold isopropanol was added. Tubes were inverted several times for DNA to precipitate and centrifuged at 7,647 g for 15 min. The pellet was retained and washed with 400 µL of ice-cold ethanol and centrifuged at 7,647 g for 5 min. The pellet was air-dried for 10 min and re-suspended in 50 µL in low-TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.5) to dissolve DNA and stored at -20 ℃.

Two gene regions, β-tubulin 2 (TUB2) [(BT2a5'-GGTAACCAAATCGGTGCTTTC-3'), (BT2b5'-ACCCTCAGTGTAGTGACCCTTGC3')] (Glass and Donaldson, 1995), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [(GD92F1 5'-GCCGTCAACGACCCCTTCATTGA-3'), (GDR1 5'-GGGTGGAGTCGTACTTGA GCATGT-3')] (Templeton *et al*., 1992) and internal transcribed spacer of the ribosomal DNA (ITS) [(ITS-1F5'-CTTGGTCATTTAGAGGAAGTAA-3')(Gardes and Bruns, 1993)], [(ITS-4 5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990)] were amplified.

All PCR amplifications were carried out, as described by Weir *et al*. (2012). The PCR products were sequenced for both directions using Applied Biosystems, 3500 Genetic Analyzer at the Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya, Sri Lanka.

Pathogenicity test

Anthracnose lesions in ripe avocado fruits collected in the study were examined, and the symptoms were recorded. Isolates of *C. endophytica* and *C. siamense* were grown on pure culture. Freshly harvested fruits of uniform size, devoid of blemishes or any disease symptoms, were chosen for artificial inoculation. Suspensions of conidia of an isolate each of *C. endophytica* and *C. siamense* were prepared by scraping mycelium, suspending them in sterile distilled water and filtering through glass wool. The concentration of conidia was adjusted to 1 x 10^6 mL^{-1}. Four drops (20 µL) of conidia from each isolate were applied on to four equally distanced sites along the fruit surface, from the stem-end to the blossom-end. Six replicate fruits were used for each isolate. The fruits treated with drops of SDW were maintained as controls. Inoculated and control fruits were incubated in separate trays, lined with moistened tissues, and covered with glass plates, at 28 - 30 ℃. The fruits were examined daily and the symptoms, when appeared, were

compared with those of the original diseased fruits in which the disease was initially observed. The pathogens were re-isolated from symptomatic fruits on PDA. Morphological features of the colonies and, asexual reproductive stages of the isolate, were compared with those of the original isolates used for inoculation.

Data analysis

The species affiliations and identities were determined through similarity-based searches of the NCBI GenBank Database (htttp://www.ncbi.gov). Based on the identifications that resulted from the BLAST search, a combined phylogenetic analysis for ITS, TUB2, and GAPDH was conducted including the authenticated sequences of the members belonging to the *C. gloeosporioides* complex obtained from the GenBank (Table 1). Bayesian inference analysis was performed for the combined matrix. The best fitting substitution model was determined with jModelTest v.2 (Darriba *et al*., 2012) using the Akaike information criterion. The nucleotide substitution model General Time Reversible was selected. Bayesian inference was conducted to obtain posterior probabilities using MrBayes ver. 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with 10,000,000 generations Markov chain Monte Carlo chains with a sampling frequency of every 1,000 generations. The initial 25% samples from each run were discarded as burning. A majority rule consensus tree was calculated using the remaining trees to obtain the posterior probabilities for each node. The resulting tree was visualized and edited in FigTree ver. 1.4.3 (Rambaut and Drummond, 2016). *Colletotrichum hippeastrum* (isolate CBS 241.78) was used as the out group. All the sequences, generated during the study and used in multi-gene analyses, were deposited in GenBank and the accession numbers are given in Table 1.

RESULTS

Isolation of pathogen

Colletotrichum infections in ripe fruits appeared as blackish brown, and circular lesions of different sizes with slightly irregular margins scattered over the peel of ripe fruit. Salmon colored, sticky conidia masses, resembling slimy droplets, were seen in the centre of older lesions (Figure 1). Lesions enlarged in size widening their diameter, up to 3 - 5 cm or more. Multiple infections in closer proximity tended to coalesce forming larger diseased areas.

Thirty five isolates were obtained from the anthracnose lesions in fruits collected from different locations in the Central Province where avocados are mostly produced and, also from the North Western Province of Sri Lanka. All 35 isolates produced oblong conidia, and the colonies of majority of the isolates consisted of pink conidial masses. The isolates were identified to the Genus *Colletotrichum* from their cultural and conidial characteristics.

Phylogenetic analyses

The combined data set for ITS, TUB2, and GAPDH sequences consisted of 1286 bps. The phylogenetic tree

Figure 1: Anthracnose symptoms of ripe avocado. (**a**) external symptoms of a fruit with multiple lesions; (**b**) a lesion with pinkish conidiomata, and (**c**) vertically halved fruit through a lesion.

that resulted from the Bayesian analysis is given in the Figure 2. All members of the *C. gloeosporioides* complex formed a monophyletic group while all the Sri Lankan *Colletotrichum* isolates identified as *C. siamense* and *C. endophytica* formed a separate monophyletic clade. However, the both clades received low support, posterior probability of 0.84 and 0.73 respectively and the clades are unresolved.

Morphological characteristics of *Colletotrichum siamense*

The colonies on PDA first appeared white and turned pale yellow to grey with time. Aerial mycelium was greyish white, dense, wooly, or cottony with very few conidial masses at the center. Sectoring was observed in some cultures. Conidia were cylindrical with slightly rounded ends and sometimes tapering towards one end and measured 20.8 - 30.4 µm × 7.0 - 8.4 µm. Appressoria were ovoid or irregularly lobed, 9.2 - 11.1 µm in diameter. Some cultures produced both ovoid and lobed appressoria while others produced only ovate. Appressoria colour ranged from brown to dark brown (Figure 4).

Morphological characteristics of *Colletotrichum endophytica* isolate

Colonies on PDA first appeared white and the center of colony of some cultures became grey to ash color with time. Aerial mycelium at the periphery was white, dense, wooly or cottony with numerous conidia masses. Some isolates produced sectoring after sub-culturing. Conidia

Table 1: Accession numbers of authenticated sequences of the *C. gloeosporioides* species complex obtained from the GenBank and the sequences generated from the present study, used for phylogenetic analysis.

Species	Isolate	Host	ITS	TUB2	GAPDH	References
C. aenigma	ICMP 18608*	*Persea americana,* Israel	JX010244	JX010389	JX010044	Weir *et al*. (2012)
C. aeschynomenes	ICMP 17673*	*Aeschynomene virginica,* USA	JX010176	JX010392	JX009930	Weir *et al*. (2012)
C. alatae	CBS 304.67*	*Dioscorea alata,* India	JX010190	JX010383	JX009990	Weir *et al*. (2012)
C. alienum	ICMP 12071*	*Malus domestica,* New Zealand	JX010251	JX010411	JX010028	Weir *et al*. (2012)
C. aotearoa	ICMP 18537*	*Coprosma sp.,* New Zealand	JX010205	JX010420	JX010005	Weir *et al*. (2012)
C. asianum	ICMP 18580*	*Coffea arabica,* Thailand	FJ972612	JX010406	JX010053	Weir *et al*. (2012)
C. camelliae	ICMP 10643	-	JX010224	JX010436	JX009908	Weir *et al*. (2012)
C. chengpingense	MFLUCC 15 0022*	-	KP683152	KP852490	KP852469	Jayawardena *et al*. (2020)
C. clidemiae	CMP 18658*	*Clidemia hirta,* USA, Hawaii	JX010265	JX010438	JX009989	Weir *et al*. (2012)
C. conoides	CAUG17*	-	KP890168	KP890174	KP890162	Jayawardena *et al*. (2020)
C. cordylinicola	MFLUCC 090551*	*Cordyline fruticosa,* Thailand	JX010226	JX010440	JX009975	Weir *et al*. (2012)
C. endophytica	CAUG28	*Capsicum annuum,* China	KP145441	KP145469	KP145413	Diao *et al*. (2017)
	UPBT_CE01	*Persea americana,* Sri Lanka	MG786653	MG981211	MG981232	Present study
	UPBT_CE02	*Persea americana,* Sri Lanka	MG786654	MG981212	MG981233	Present study
	UPBT_CE03	*Persea americana,* Sri Lanka	MG786656	MG981213	MG981234	Present study
	UPBT_CE04	*Persea americana,* Sri Lanka	MG786658	MG981214	MG981235	Present study
	UPBT_CE05	*Persea americana,* Sri Lanka	MG786659	MG981215	MG981236	Present study
	UPBT_CE06	*Persea americana,* Sri Lanka	MG786660	MG981216	MG981237	Present study
	UPBT_CE07	*Persea americana,* Sri Lanka	MG786661	MG981217	MG981238	Present study
	UPBT_CE08	*Persea americana,* Sri Lanka	MG786662	MG981218	MG981239	Present study
	UPBT_CE09	*Persea americana,* Sri Lanka	MG786663	MG981219	MG981240	Present study
C. fructivorum	Coll1414 *	-	JX145145	JX145196	--	Jayawardena *et al*. (2020)
C. fructicola	ICMP 18581*	*Coffea arabica*, Thailand	JX010165	JX010405	JX010033	Weir *et al*. (2012)
C. gloeosporioides	IMI 356878*	*Citrus sinensis*, Italy	JX010152	JX010445	JX010056	Weir *et al*. (2012)
C. grevilleae	CBS 132879	*Grevillea* sp.	KC297078	KC297102	KC297010	Liu *et al*. (2016)

C. grossum	CAUG 7*	Chili pepper	KP890165	KP890171	KP890159	Diao *et al*. (2017)
C. hebeiense	MFLUCC13–0726*	-	KF156863	KF288975	KF377495	Jayawardena *et al*. (2020)
C. henanense	CGMCC 3 17354*	-	KJ95510 9	KJ955257	KJ954810	Jayawardena *et al*. (2020)
C. hippeastrum	CBS 241.78	*Hippeastrum* sp., Netherlands	JX010293	--	JX009932	Weir *et al*. (2012)
C. horii	NBRC 7478*	*Diospyros kaki*, Japan	GQ329690	JX010450	GQ329681	Weir *et al*. (2012)
C. jiangxiense	CGMCC 3.17363*	-	KJ955201	KJ955348	KJ954902	Jayawardena *et al*. (2020)
C. kahawae sub sp. ciggaro	CBS 237.49*	*Hypericum perforatum,* Germany	JX010238	JX010432	JX010042	Weir *et al*. (2012)
C. kahawae sub sp. kahawae	IMI 319418*	*Coffea arabica*, Kenya	JX010231	JX010444	JX010012	Weir *et al*. (2012)
C. musae	CBS 116870*	*Musa* sp., USA	JX010146	HQ596280	JX010050	Weir *et al*. (2012)
C. nupharicola	CBS 470.96*	*Nuphar lutea subsp.polysepala,* USA	JX010187	JX010398	JX009972	Weir *et al*. (2012)
C. perseae	CBS 141365*	*Persea americana,* Israel	KX620308	KX620341	KX620242	Sharma *et al*. (2017)
C. psidii	CBS 145.29*	*Psidium* sp., Italy	JX010219	JX010443	JX009967	Weir *et al*. (2012)
C. queenslandicum	ICMP 1778*	*Carica papaya*, Australia	JX010276	JX010414	JX009934	Weir *et al*. (2012)
C. rhexiae	Coll 1026*	-	JX145128	JX145179	--	Ma *et al*. (2018)
C. salsolae	ICMP 19051*	*Salsola tragus,* Hungary	JX010242	JX010403	JX009916	Weir *et al*. (2012)
C. siamense	UPBT_CS11	*Persea americana,* Sri Lanka	MG786651	MG981245	MG981220	Present study
	UPBT_CS12	*Persea americana,* Sri Lanka	MG786652	MG981246	MG981221	Present study
	UPBT_CS13	*Persea americana,* Sri Lanka	MG786655	MG981247	MG981222	Present study
	UPBT_CS14	*Persea americana,* Sri Lanka	MG786657	MG981248	MG981223	Present study
	UPBT_CS15	*Persea americana,* Sri Lanka	MG786664	MG981249	MG981224	Present study
	UPBT_CS16	*Persea americana,* Sri Lanka	MG786667	MG981250	MG981225	Present study
	ICMP 18578*	*Coffiea Arabica,* Thailand	JX010171	JX010404	JX009924	Weir *et al*. (2012)
C. temperatum	Coll883 *	-	JX145159	JX145211	-	Jayawardena *et al*. (2020)
C. theobromicola	MUCL 42294*	*Stylosanthes viscosa,* Australia	JX010289	JX010380	JX009962	Weir *et al*. (2012)
C. ti	ICMP 4832*	*Cordyline* sp., New Zealand	JX010269	JX010442	JX009952	Weir *et al*. (2012)
C. tropicale	CBS 124949*	*Theobroma cacao*, Panama	JX010264	JX010407	JX010007	Weir *et al*. (2012)
C. wuxiense	JS1A32	-	KU251591	KU252200	KU252045	Wang *et al*. (2016)
C. xanthorrhoeae	BRIP 45094*	*Xanthorrhoea preissii*, Australia	JX010261	JX010448	JX009927	Weir *et al*. (2012)

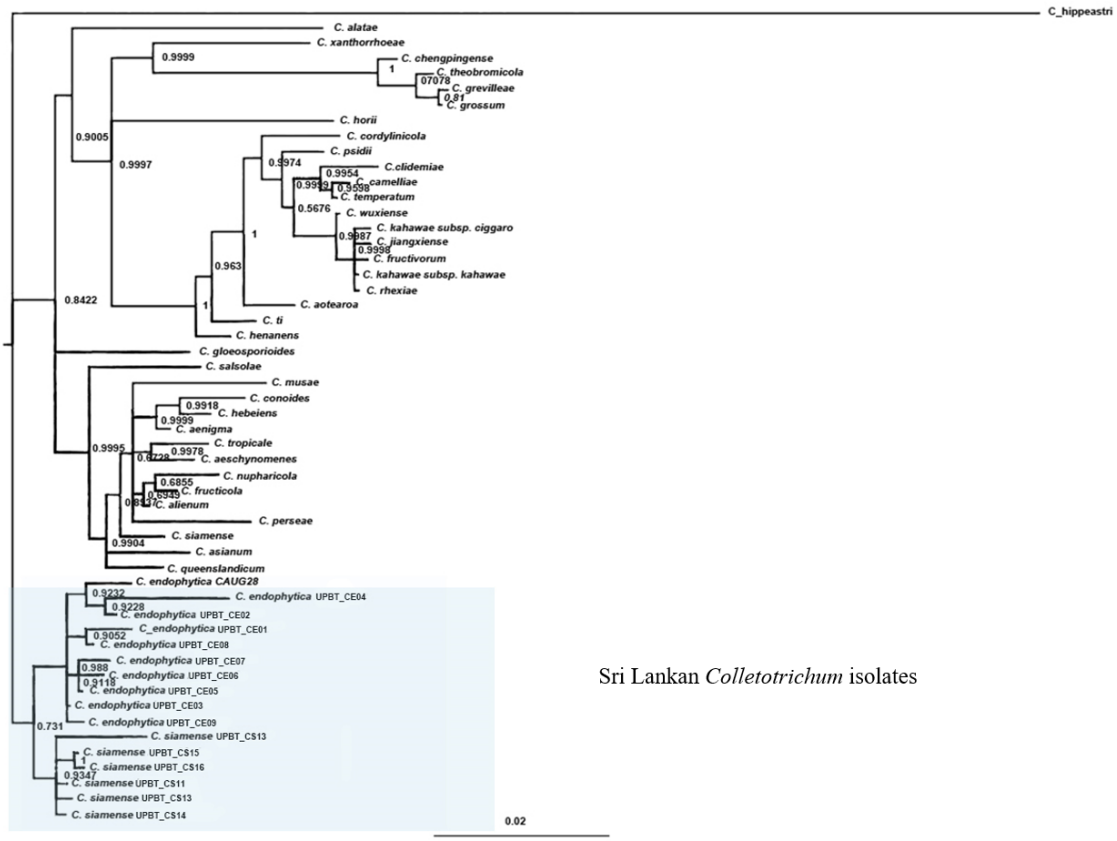

Figure 2: Bayesian inference phylogenetic tree of *Colletotrichum* isolates based on ITS, TUB2, and GAPDH sequences of the present study together with other authentic culture sequences; Bayesian posterior probability values ≥ 0.5 are shown at the nodes.

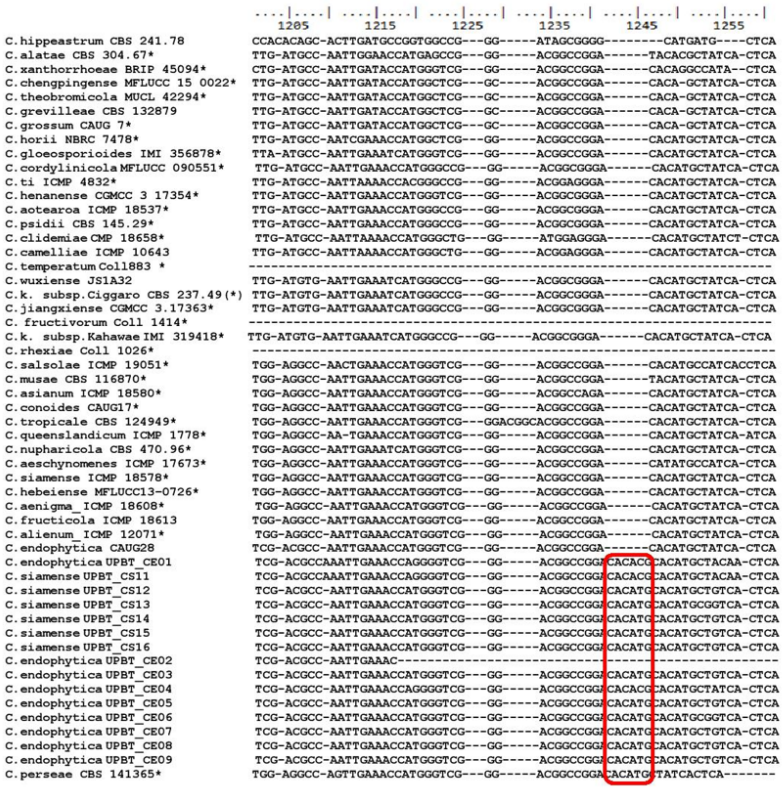

Figure 3: A part of the alignment of the GAPDH gene region showing the six-base pair INDEL of 15 Sri Lankan isolates and *C. perseae*, being either CACACG or CACATG.

were cylindrical with slightly rounded ends. Length and breadth of the conidia varied from 20.7 - 32.6 µm (length) and 6.9 - 9.9 µm (breadth). Appressoria were ovoid or irregularly lobed. All cultures produced both ovoid and lobed appressoria. Appressoria were initially pale brown color and later turned dark brown (Figure 5).

Pathogenicity test

The two *Colletotrichum* species could be repeatedly isolated from diseased avocados in the study, indicating their consistent presence in infected fruits. Healthy fruits, artificially inoculated with the two fungi, developed typical anthracnose symptoms that were observed originally, 6 -7 days after inoculation. The control fruits did not develop any disease symptoms. Morphological characteristics of the colony and conidia of the two fungi re-isolated were similar to those of the original isolates used for inoculation.

DISCUSSION

Colletotrichum gloeosporioides (Sivanathan and Adikaram, 1989) and *C. acutatum* (Silva-Rojas and Ávila-Quezada, 2011) have been believed for decades to be the pathogens causing anthracnose disease in avocado and, certain other tropical and sub-tropical fruit species. The two species show morphological similarities. The conidia morphology of *C. acutatum* being the only distinguishable, but often inconsistent, character between them. Morphology and the development of reproductive structures have been utilized in the characterization of the genus *Colletotrichum* and its teleomorph, *Glomerella,* by taxonomists. The variability of morphological characters with changing environmental and growth conditions makes them unreliable as taxonomical criteria. Molecular-based methods are presently considered advantageous in the species level identification of the genus *Colletotrichum* (Weir *et al*., 2012) than morphological features. In general, morphological differences among isolates within the genus *Colletotrichum* do not correlate with molecular differences.

The present study used multigene sequence analysis with two coding genes, TUB2, GAPDH, and the nuclear ITS region that contributes to a higher resolving ability for species level identification of *Colletotrichum* causing avocado anthracnose in Sri Lanka. Two species, *C. endophytica* and *C. siamense*, were identified as casual agents. ITS region has been useful only for the identification of *Colletotrichum* isolates into the species complex level (Prihastuti *et al*., 2009). *Colletotrichum endophytica*, belonging to the *C. gloeosporioides* species complex, was isolated as an endophyte in *Pennisetum purpureum*. *Colletotrichum endophytica* was later reported as causing anthracnose disease in *Camellia sinensis* (Wang *et al*., 2016) and chili in China, black pepper in India (Chethana *et al*., 2015) and more recently in mango also in Southern China (Li *et al*., 2019).

TUB2 sequences, generated for *C. endophytica* in the present study and deposited in the GenBank, would therefore be a valuable source of reference sequence material for future studies of *Colletotrichum*. The present study did not encounter either *C. gloeosporioides* or *C. gigasporum* that were previously identified to be associated with the avocado anthracnose in Sri Lanka (Hunupolagama *et al*., 2015).

Interestingly, the authenticated isolate of *C. endophytica* [CAUG28 (Diao *et al*., 2017)] was also grouped, within the *C. endophytica* clade, together with the Sri Lankan isolates. However, the authenticated *C. siamense*, which is also an ex-type (ICMP_18578) Weir *et al*. (2012), grouped in the main clade with the other species of the *C. gloeosporioides* complex, separate from the Sri Lankan *C. siamense* isolates.

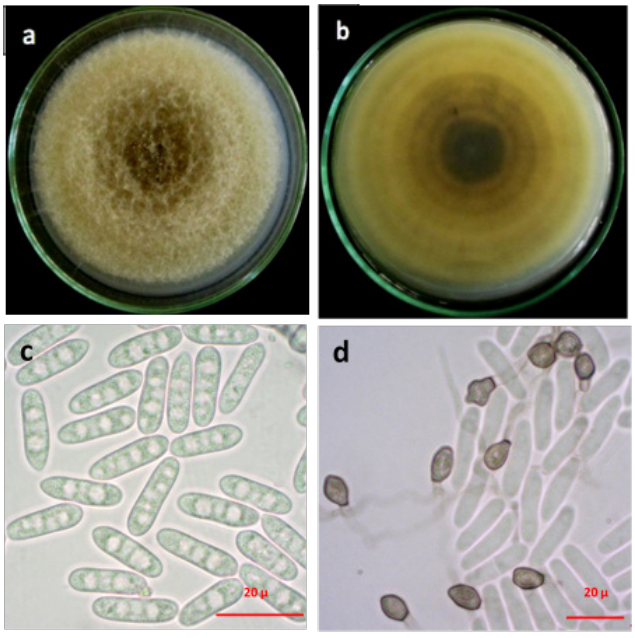

Figure 4: A 10 - day old colony of *Colletotrichum siamense*, (a) upper surface, (b) lower surface, (c) conidia, and (d) appressoria.

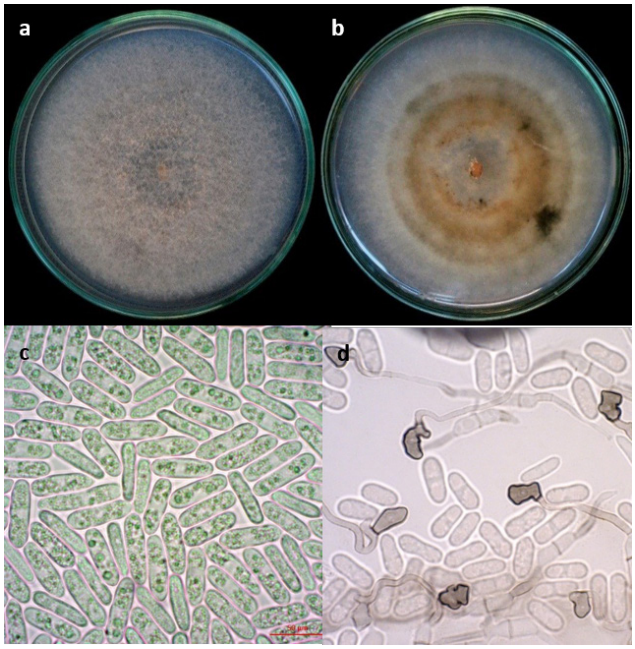

Figure 5: A 10 - day old colony of *Colletotrichum endophytica* on PDA, (a) upper surface, (b) lower surface, (c) conidia, and (d) appressoria.

Based on multi-locus phylogenetic analyses, eight previously described species and a novel species (*C. perseae*) were identified as avocado anthracnose pathogens in Israel (Sharma *et al*., 2017). In addition, several more *Colletotrichum* species were reported causing anthracnose disease in avocado from countries worldwide, raising the total number of species to over fifteen. The inconsistency of the *Colletotrichum* species reported warrants further studies on the avocado-*Colletotrichum* pathosystem in avocado producing and marketing countries of the world.

Colletotrichum siamense was first described as a causal agent of coffee berry anthracnose from Northern Thailand (Prihastuti *et al*., 2009). The species was later recorded on many hosts across tropical and subtropical regions without any host specificity, peach (Yang *et al*., 2009), mango (Phoulivong *et al*., 2010; Udayanga *et al*., 2013), custard apple, *Cerbera* sp., figs, and papaya (Rampersad, 2011; Udayanga *et al*., 2013) and *Pongamia pinnata* (Dwarka *et al*., 2016). The understanding of *C. siamense* is still in a state of confusion. The present study identified variable cultural, conidial and appressorial characters within *C. siamense* suggesting that *C. siamense* might not be a single species. Molecular analysis of 85 *Colletotrichum* isolates from fruit crops in India, using *ApMat* marker, resolved *C. siamense* to be a species complex (Sharma *et al*., 2015). However, Liu *et al*. (2016), following a molecular analysis based on Genealogical Concordance Phylogenetic Species Recognition (GCPSR) and coalescent methods, concluded that *C. siamense sensu lato* is a single species rather than a species complex.

The present phylogenetic analyses have shown a separation of the Sri Lankan *C. siamense* isolates from the authenticated *C. siamense* which may supports the idea that *C. siamense* might well be a species complex (Sharma *et al*., 2015) rather than a single species. Similarly, the authenticated *C. endophytica* together with the Sri Lankan *C. endophytica* isolates are placed outside the main *C. gloeosporioides* complex, indicating the diversity of these species at the molecular level. The separation of the Sri Lankan *Colletotrichum* isolates from the rest of the species of the *C. gloeosporioides* complex indicates that the Sri Lankan isolates are genetically diverse. While studying the DNA sequence alignments, a notable difference of all 15 Sri Lankan isolates was an INDEL of six base pairs, either CACACG or CACATG, that was unique only to the local isolates in the GAPDH region (Figure 3). This INDEL was also shared by *C. perseae*, a novel species that was recently identified and described as causing avocado anthracnose disease in Israel (Sharma *et al*., 2017).

The present study reports *C. endophytica* for the first time from avocado anthracnose disease. This would necessitate new disease management strategies for avocado anthracnose since *C. endophytica* is new to avocado. This may also increase its importance as a quarantine pathogen (Yan *et al*., 2015). These re-iterate once again the importance of accurate identification of causal agents in designing disease management strategies.

CONCLUSION

In conclusion, the present study identified *C. endophytica* and *C. siamense* as pathogens avocado anthracnose where this is the first report of *C. endophytica* from avocado. The study also reports for the first time the semi-systemic nature of symptom development in the disease.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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