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# *Colletotrichum* species causing anthracnose disease in *A. andraeanum*, manifested as spathe rot also in addition to spadix rot and leaf spot.

## Running title: *Colletotrichum* spp. causing anthurium anthracnose

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**Abstract** Anthurium is an ornamental flowering plant, cultivated in tropical and sub-tropical climates. Anthracnose is a major disease in anthurium, known to be manifested as spadix rot and leaf spot, and caused by *Colletotrichum gloeosporioides* or its teleomorph, *Glomerella cingulata*. The objective of the present study was to describe spathe rot, a new disease, and identify *Colletotrichum* species associated with spathe rot, spadix rot and leaf spot, using multi-locus DNA sequence analyses. Diseased spathes were obtained from anthurium var. Tropical Red while the diseased spadix and leaves were from var. Hawaiian Butterfly. *Colletotrichum* was isolated from diseased spathe, spadix, and leaf on Potato Dextrose Agar (PDA) following surface sterilization. DNA, extracted from the isolates, was subjected to multigene DNA sequence analyses using ITS, β-tubulin and GAPDH. The two isolates from the spathe rot were identified as *C. fructicola* and *C. orchidearum* and the isolates from the spadix rot and leaf spot were identified as *C. fruiticola* and *C. siamense* respectively. Koch's postulates were performed using *C. fructicola* and *C. orchidearum*, isolated from the spathe rot, and their pathogenicity was confirmed. The manuscript reports *C. fructicola* and *C. orchidearum* as the causal agents of the new disease, the spathe rot, and the association of *C. fructicola* and *C. siamense* with the spadix rot and leaf spot respectively.

**Keywords** *Colletotrichum fructicola* · *Colletotrichum orchidearum* · ITS, β-tubulin and GAPDH · *Colletotrichum gloeosporioides* species complex · *C. orchidearum* species complex

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## Introduction

Anthurium (*A. andraeanum* Linden ex André; Family Araceae) is native to Mexico, Costa Rica, Cuba, and Brazil and widely cultivated as an ornamental in tropical and sub-tropical climates. The genus *Anthurium* has nearly 1000 species, making it the largest genus in the plant family, Araceae. Growth habits vary depending on the species, some are terrestrial, and others are epiphytic.

Most cut-flower cultivars are selections of *A. andraeanum*, growing as an epiphyte. Anthuriums produce a type of inflorescence, known as spadix, with many small flowers borne on a fleshy stem that is surrounded by a large, leaf-like, brightly colored bract, known as the spathe. Anthurium flowers have a longer

vase life of about 6 weeks or more, depending on the variety and the season. The attractive and long-lasting flower parts and ornamental leaves make anthurium popular as both a cut-flower crop and flowering potted-plant. Anthurium is one of the most commercialized cut flowers in the world.

Anthuriums are affected by several fungal and bacterial diseases. Among them are bacterial blight, caused by *Xanthomonas axonopodis* pv. *dieffenbachiae* (Aysan & Sahin, 2003), necrotic leaf and spathe spotting incited by *Pseudomonas* species (Annelle et al., 2017) and bacterial wilt (*Ralstonia solanacearum*) which could be severe especially under hot weather conditions (Norman & Ali, 2015). Root infections by Oomycota, *Phytophthora nicotianae* var. *parasitica* and *Pythium splendens*, exhibit wilting, leaf yellowing (chlorosis), and root dieback (Norman & Ali, 2015). *Rhizoctonia* species can also infect the root system. Among fungal diseases, anthracnose is the most typical fungal disease in anthurium (Abeygunawardena 1969) which was known to be caused by *Colletotrichum gloeosporioides* (Kagiwata, 1990; Aragaki et al., 1968) or its teleomorph, *Glomerella cingulata* (Abeygunawardena, 1969). Anthracnose disease is reported to be manifested as leaf spot and spadix rot or black nose (Norman & Ali, 2015). *Colletotrichum* infection of anthurium leaf causes brown, circular spots up to 2 cm diameter with a brownish yellow margin. The spadix rot or black nose can be a serious problem in cut-flower and potted-plant production during humid and warm conditions. Studies have shown a wide variation in resistance and susceptibility among cultivars to anthracnose pathogen (Aragaki et al., 1968) and some accessions have shown outstanding resistance.

Genus *Colletotrichum* includes many important plant pathogenic fungi and is best known for causing anthracnose disease on a wide range of fruit, vegetable, and ornamental hosts, especially in subtropical and tropical regions (Guarnaccia et al., 2021). In recent years, there were frequent reports on the identity of the *Colletotrichum* species associated with anthracnose disease in numerous fruit, vegetable and ornamental plants using multi-gene sequencing approach. Past phylogenetic analyses, using multiple sequence data, revealed that the genus *Colletotrichum* comprises of 14 species complexes, and over 20 singletons. *Colletotrichum gloeosporioides* species complex is collective of *C. gloeosporioides* sensu stricto and, 52 allied species (Jayawardena et al., 2020). Similarly, *C. acutatum* is now considered a species complex harboring 40 closely related species (Jayawardena et al., 2020). Damm et al., (2019) erected *orchidearum* species complex, which includes ten species.

A molecular detection method, based on differences in ITS sequences of *Colletotrichum* genus, was developed to detect anthracnose infections in anthurium (Xing et al., 2008). However, the *Colletotrichum* species associated with anthracnose disease in the spadix or the leaf of anthurium have not been accurately identified using high resolution protein sequence data. There were no previous reports of the occurrence of anthracnose disease in the spathe of anthurium. The objective of the present study was to describe the spathe rot, a new disease of anthurium, and investigate the *Colletotrichum* species associated with spathe rot, spadix rot or black nose and leaf spot using multigene DNA sequencing.

## Materials and methods

### Examination of diseased specimens and isolation of pathogen/s

Inflorescences of two varieties of anthurium (*A. andraeanum*), showing spathe rot (var. Tropical Red), spadix rot with black nose symptoms and leaf spot (var. Hawaiian Butterfly), were collected from Peradeniya and Dodanwala area (Kandy District, Central Province of Sri Lanka) respectively. Infected inflorescences were placed in sealed polythene bags and taken to the Plant Pathology laboratory at the Department of Botany, University of Peradeniya. Small segments (0.5 × 0.5 $cm^2$) cut from the advancing margin of the lesions of the spathe, spadix and the leaf were separately surface sterilized using 3% sodium hypochlorite for 2 min. Tissue segments were blot-dried on sterile filter paper and placed on Potato Dextrose Agar (PDA) medium. The plates were incubated at room temperature (28 ± 2 °C) for 5 days. The fungi were sub-cultured by transferring discs of mycelium advancing from the diseased tissue segments on to fresh PDA plates. Five isolates, ANT1 (from infected spathe), ANT2 (infected spadix), ANT3 & ANT4 (infected leaf) and ANT5 (infected spathe) that showed colonies, conidial masses, or conidia with morphological features typical to *Colletotrichum* were taken for species level identification using morphological and molecular characteristics.

### Preparation of single-spored cultures

A suspension of spores from each isolate was prepared by scraping mycelium from 10-day old cultures, suspending in a 5 ml portion of sterile distilled water and filtering through a sterile muslin cloth. The concentration of spores in the filtrate was adjusted to ($3 \times 10^5$ $ml^{-1}$). Aliquots (100 μl) were aseptically poured over a thin layer of 1% tap water agar in glass petri plates. The plates were incubated in an inclined position at RT (28 ± 2 °C) for 18–24 h. The reverse side of tap water agar plates was examined under the objective lens of light microscope (Olympus CX22) and a single germinating conidium was located and marked. A disc (6 mm diameter) of agar containing a germinated spore was cut and transferred on to fresh PDA medium (Johnston & Booth, 1983). The plates were incubated at RT (28 ± 2 °C) for 7–10 days and the colonies developed were sub-cultured on PDA.

### DNA extraction, PCR amplification and sequencing

Mycelium scraped from fresh cultures grown on PDA with a sterile pipette tip was used for the DNA extraction which was performed using Wizard® genomic DNA purification kit (Promega Corporation, USA), according to the manufacturer's protocol with modifications (addition of proteinase K). The quality and quantity of DNA were estimated visually by 2% agarose gel electrophoresis stained with ethidium bromide. Current taxonomic and phylogenetic studies on the genus *Colletotrichum* recommends the use of multiple gene regions in the identification to the species level (Damm et al., 2012). The present study used three loci, internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin (TUB) and were amplified using primer pair ITS1 and ITS4, BT2a and BT2b and GD92F1 and GDR1, respectively (White et al., 1990; Templeton et al., 1992; Gardes & Bruns, 1993; Glass & Donaldson, 1995; Weir et al., 2012).

PCR reactions were performed using Applied Biosystems Veriti 96-well thermal cycler (9902, Singapore). Amplification reactions had a total reaction volume of 40 μL which consisted of 1× PCR buffer (Promega Corporation, USA), 5.6% DMSO (v/v), 40 μM dNTPs (Promega Corporation, USA), 1.5 mM $MgCl_2$, 0.2 μM of each forward and reverse primers, 0.25 U of GoTaq™ *Taq* DNA polymerase (Promega Corporation, USA), sterilized water and genomic DNA.

The thermal programme that was used to amplify the gene regions is as follows; except for the annealing temperature, the other conditions remained same for all regions; 4 min at 95 °C, followed by 35 cycles of denaturation (94 °C for 30 s), annealing (30 s at 52 °C for ITS; 60 °C for GAPDH and 55 °C for TUB), elongation (72 °C for 90 s), and a final 7 min extension at 72 °C and cooling down step to 4 °C. PCR products were examined by electrophoresis on a 2% agarose gel, stained with ethidium bromide. Amplicons were sequenced for both directions (Applied Biosystems, 3500 genetic analyzer) at the Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya.

### Data analysis

Initial blast results showed that our isolates belong to the *Colletotrichum gloeosporioides* and *C. orchidearum* species complexes. Therefore, two combined phylogenetic analyses based on ITS+GAPDH+TUB genes were carried out to estimate the placement of our isolate within the two species complexes. The sequences generated in this study were supplemented with additional sequences obtained from the GenBank. Multiple sequence alignments were generated with MEGA v.7.0.26 (Kumar et al., 2016) and improved manually. The maximum likelihood (ML) analyses were conducted using RAxML v8.2.10 (Stamatakis, 2014) performed on the CIPRES Science Gateway server (Miller et al., 2012) and using the GTR + GAMMA model inferred using partitionfinder v. 1.1.1 (Lanfear et al., 2012). Branch support values were determined using the rapid bootstrapping algorithm with 1000 replicates. The phylogenetic trees were visualised in FigTree v. 1.4.3 (Rambaut & Drummond, 2016) and edited in Adobe Illustrator CC 2018.

### Koch's postulates

Fungi were isolated from anthracnose lesions and grown in pure culture as described previously. Twelve potted anthurium plants var. Tropical Red at a uniform stage of flowering, devoid of any blemishes or disease symptoms, were used for artificial inoculation experiments. Suspensions of conidia of the two isolates of *C. fructicola* (ANT1) and *C. orchidearum* (ANT5) were prepared and the concentration of conidia was adjusted to $1 \times 10^6$ $ml^{-1}$. Four drops (20 μl) of conidia were

applied on to the upper surface of spathe (two drops on each vertical half) and four spathes were used for each isolate. Drops of sterile distilled water were applied on to four spathes as controls. The inoculated and control spathes were kept covered with perforated polythene bags at 28–30 °C and after 24 h the polythene bags were taken off. The inoculated spathes were examined regularly and the symptoms, when appeared, were described, and compared with those of the symptoms that were observed in the original diseased spathes from which the pathogens were isolated. The pathogen was re-isolated from the symptomatic spathes on PDA. The colony's morphological features and the asexual reproductive stages of the isolate were compared with those of the original isolate used for inoculation.

## Results

### Spathe rot symptoms

*Colletotrichum* infection of the brightly colored spathe of anthurium was observed for the first time in the present study. Symptoms appeared on the upper surface of the spathe (*A. andraeanum* var. Tropical Red) as circular or irregularly shaped, medium-sized, 1–2 cm diameter, isolated lesions with wavy margins. Individual lesions had a straw color periphery and brownish-black center (Fig. 1). On closer examination, the centre of the lesions was found to have two or more concentric rings. There were numerous isolated individual lesions, distributed over a single spathe, more densely in the two upper lobes than the lower portion. Sometimes two or more individual lesions tended to coalesce to form larger diseased areas.

### Spadix rot

The initial symptoms of spadix rot were observed as small, brown to black color flecks on the middle portion of the floral spadix. The flecks rapidly enlarged to form a brown to black and 2–3 mm wide ring on the middle portion of the infected spadix (Fig. 2). The healthy area is sharply demarcated from the diseased ring. The ring of diseased tissue expanded further on both directions, more rapidly downwards, and encompassed almost the entire spadix within 2 days (Fig. 2). The diseased spadix eventually fell off.

### Molecular identification

After exclusion of ambiguously aligned regions from the *C. gloeosporioides* species complex, the final dataset included 47 isolates with an alignment length of 1287 characters (ITS = 484, GAPDH = 287 and TUB = 446) including gaps. The best scoring RAxML tree resulted with a Likelihood value of −4670.274700. Two isolates (ANT3 and ANT4) isolated from leaves clustered with *C. siamense* (Fig. 3), while two isolates from spathe (ANT1) and spadix (ANT2) clustered with *Colletotrichum fructicola*.

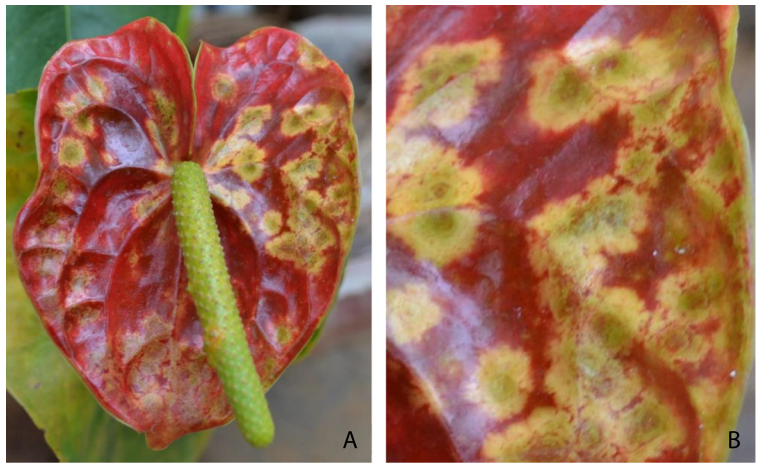


**Fig. 1** (A) Anthracnose lesions in the spathe of anthurium inflorescence var. Tropical Red. The infected areas represent straw color periphery with wavy margin and brownish center. (B) A portion of the infected spathe enlarged


**Fig. 2** (A) Spadix rot or black nose of anthurium variety Hawaiian Butterfly at initial, and (B) advanced stage. (C) Affected middle portion of the inflorescence expanded vertically into a darkened area of florets, and (D) subsequent ash color fungal growth

Phylogenetic analyses

The combined ITS, GAPDH and TUB alignment was used to resolve the species relationship in the *Colletotrichum orchidearum* species complex (Fig. 4). The alignment comprised 21 strains (including the outgroup taxon *Colletotrichum magnum* (CBS 519.97) and the manually adjusted dataset comprised 1214 characters (ITS = 547, GAPDH = 172 and TUB = 495) including gaps. The best scoring RAxML tree resulted with a Likelihood value of −2815.621776. Phylogenetic results showed the strain (ANT5) isolated from spathe clustered with the GenBank isolates of *Colletotrichum orchidearum.* The GenBank accession numbers obtained for the sequences generated using ITS, TUB and GAPDH for *Colletotrichum* isolates in this study are given in the Table 1.

Koch's postulates

*Colletotrichum* could be repeatedly isolated from diseased spathes of anthurium, indicating its consistency in anthracnose lesions and grown on pure cultures. Healthy spathes, artificially inoculated with each fungus, showed anthracnose lesions, within 7–10 days of incubation. There were no symptoms observed in the control spathes. Cultural and morphological characteristics of conidia of the two fungi obtained from re-isolations were similar to those of the two isolates used for inoculation.

## Discussion

The earliest record of anthracnose disease of anthurium was by Fischer (1925) who reported *Gloeosporium minutum* as a rare pest of anthurium cultures. Twenty years later, Neergaard (1942-1945) recorded *C. anthurii* (syn. *Gloeosporium anthurii*) on the foliage of *A. scherzerianum*. Subsequently, Aragaki et al., (1968) reported the spadix rot of *A. andraeanum* caused by *C. gloeosporioides.* Following a more recent study, Norman and Ali (2015) claimed that *Colletotrichum*

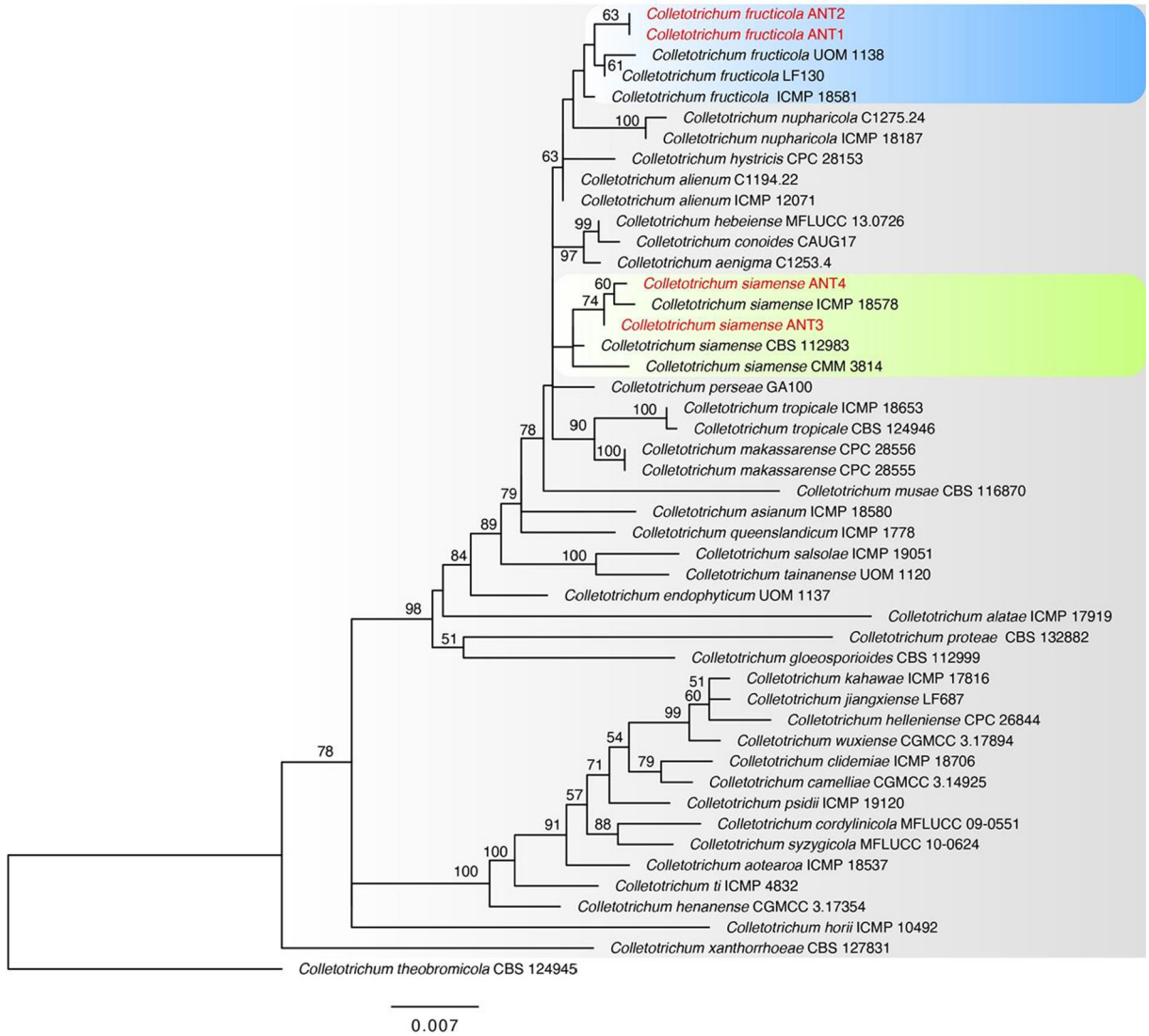


**Fig. 3** Maximum likelihood tree resulting from a RAxML analysis of the combined ITS+GAPDH+TUB alignment of the analyzed taxa in *C. gloeosporioides* species complex. RAxML bootstrap support values are given at the nodes and newly introduced strains are in red. The strain accession numbers follow the species name. The tree is rooted to *C. theobromicola* (CBS 124945)

was highly specific in attacking only the spadix portion or the nose of the *Anthurium* flower. Abeygunawardena (1969) noted that both leaf and the spadix of anthurium are infected by *Glomerella cingulata*, the sexual morph of *C. gloeosporioides*. From the findings reported above, the anthurium anthracnose could be recognized in a somewhat broader manner as a disease that affects the leaves and the spadix.

The present study reports the manifestation of anthracnose symptoms also as the spathe rot. Spathe is the most colorful and attractive component, immediately surrounding the spadix of anthurium. Botanically, the colorful spathe is a large sheathing bract enclosing the flower cluster or the anthurium inflorescence, although it is often mistaken for a flower part. This means that the anthracnose disease directly affects the floral parts, the spadix (inflorescence) and the spathe (bract but considered a floral part), and the vegetative parts, the foliage. The disease can drastically reduce their visual quality and durability, often making cut flowers and flowering potted plants unmarketable. The flowers and flowering pot plants of anthurium are popular among flower

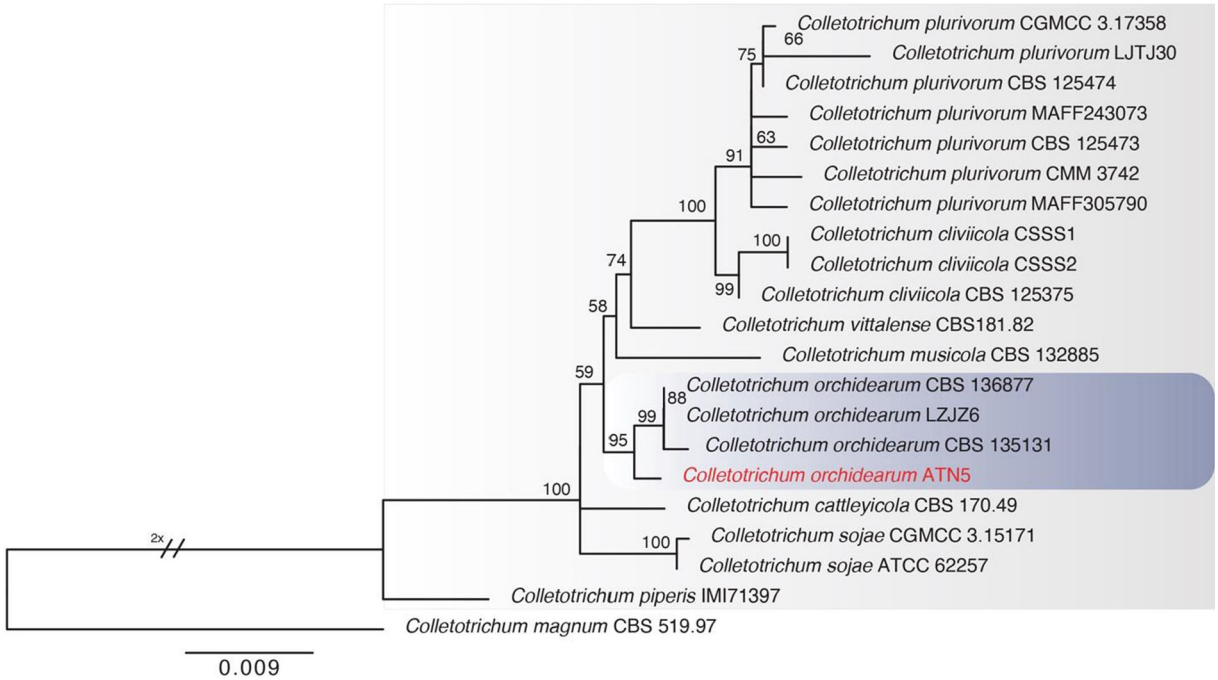


**Fig. 4** Maximum likelihood tree resulting from a RAxML analysis of the combined ITS+GAPDH+TUB alignment of the analyzed taxa in *C. orchidearum* species complex. RAxML bootstrap support values are given at the nodes and newly introduced strains are in red. The strain accession numbers follow the species name. The tree is rooted to *Colletotrichum magnum* (CBS 519.97)

arrangers because of their attractive colors, increased vase life and long-lasting qualities. The spathe rot of anthurium had either been overlooked, despite its importance, or not observed in the past. The spathe is the most prominent part of the whole structure. This study of spathe rot involved molecular identification, and the phylogeny also, of the causal agents.

Five isolates of *Colletotrichum*, collectively obtained from the spathe rot, spadix rot and leaf spot, were subjected to multi-locus phylogenetic analysis using three loci, ITS, GAPDH and TUB. The isolates obtained from the spathe rot were identified as *C. fructicola* and *C. orchidearum* while the single isolate obtained from the spadix was found to be *C. fructicola.* Both isolates from the leaf spot were identified to be *C. siamense*, belonging to the *C. gloeosporioides* complex (Weir et al., 2012). This is the only species level identification study of *Colletotrichum* causing anthracnose disease in the flower and vegetative parts of anthurium. None of these species were reported before from anthurium anthracnose.

*Colletotrichum orchidearum*, belonging to the *C. orchidearum* species complex, was first described

**Table 1** GenBank accession numbers, isolate number, species name and part of the anthurium plant from which the isolates were obtained.

| Isolate | Species | Plant part | GenBank accessions | | |
|---|---|---|---|---|---|
| | | | ITS | GPDH | TUB |
| ANT1 | *C. fructicola* | Spathe | – | MW390917 | MW390922 |
| ANT2 | *C. fructicola* | Spadix | MW391578 | – | MW390921 |
| ANT3 | *C. siamense* | Leaf | MW391580 | MW390919 | – |
| ANT4 | *C. siamense* | Leaf | MW391579 | MW390918 | MW390923 |
| ANT5 | *C. orchidearum* | Spathe | MW391581 | MW390920 | MW390924 |

by Allescher (1902) on three different Orchidaceae species. The fungus is known as a causal agent of anthracnose of the family Orchidaceae. Farr and Rossman (2017) isolated *C. orchidearum* from numerous Orchidaceae hosts from Asian, African, and Latin American countries. Isolation of *C. orchidearum* from *Monstera deliciosa* (Araceae) from China (Yang et al., 2011) is noteworthy as *M. deliciosa* also belongs to Araceae, the family to which anthurium also belongs. Xu et al., (2016) also reported *C. orchidearum* from *A. lappa* (Asteraceae) in China.

*Colletotrichum fructicola* and *C. siamense* were first described from coffee berries in Northern Thailand (Prihastuti et al., 2009). Both species cause diseases on a broad range of plants. *Colletotrichum fructicola* was reported from anthracnose disease in two ornamental plants with medicinal properties also, *Camellia chrysantha*, a traditional Chinese specialty flower plant which is known as the "golden camellia" (Zhao et al., 2021) and *Crinum asiaticum*, grown in tropical and subtropical regions of Asia (Qing et al., 2020). *Colletotrichum siamense* was reported causing anthracnose disease in tropical ornamental plants including, *Monstera deliciosa* (Liu et al., 2021), *Zinnia elegans* (Li et al., 2021), *Erythrina crista-galli* (Li et al., 2021) and *Begonia* species (Wickramasinghe et al., 2020).

*Colletotrichum* isolates from fruit rots of blueberry and strawberry in mixed-fruit orchards in Kentucky were compared with apple isolates using morphological characteristics, gene sequences, whole genomes, and cross-inoculation trials. Seven morphotypes identified, corresponded with phylogenetic species, *Colletotrichum fructicola*, *C. fioriniae*, *C. nymphaeae*, and *C. siamense.* All blueberry isolates belonged to *C. fioriniae* and most strawberry isolates were *C. nymphaeae* with a few identified as *C. siamense* and *C. fioriniae*. All three species caused fruit rot on apples in Kentucky. Cross-inoculation studies on detached apple, blueberry, and strawberry fruits showed that all *Colletotrichum* species were pathogenic on three hosts suggesting that mixed-fruit orchards may facilitate cross-infection, creating significant management problems (Eaton et al., 2021).

A more recent molecular study conducted in Brazil by Chaves et al., (2020) reported *C. theobromicola* as the causal agent of anthracnose in the flower of anthurium, using partial sequences of the ITS, GAPDH, calmodulin, chitin synthase and actin regions. The exact part of the flower from which the fungus was isolated was not clear.

Identification of *Colletotrichum* species has traditionally been based on cultural and reproductive morphology, physiological characters and sometimes the type of host species (Muñoz et al., 2000) which has over the years led to numerous ambiguities in the taxonomy of the genus *Colletotrichum.* Identification of the genus *Colletotrichum* at the species level is difficult due to overlapping morphological characters and low genetic divergence within different species complexes (Cannon et al., 2012). The use of molecular data and, more recently the multi-gene sequence analysis, resulted in a dramatic change in the taxonomy of the genus *Colletotrichum* and, also of *C. gloeosporioides* (Penz.) Penz. & Sacc. (Cai et al., 2011; Jayawardena et al., 2020).

*Colletotrichum* species produce millions of conidia on infected anthracnose lesions which serve as a source of inoculum for fresh infections. Conidia are dispersed mainly by splashing water during rain or overhead watering. Strict sanitation is necessary to reduce build-up of inoculum, which will serve as sources of inocula within anthurium production facilities. The disease is most severe during warm and humid conditions (Norman & Ali, 2015). Accurate identification and knowledge of the species infecting anthurium flower are important not only in managing disease but also in determining its distribution, biosecurity, and its detection in flowers produced for the export market.

## Conclusions

The present study describes the spathe rot as a new disease, manifested as anthracnose symptoms in anthurium (*A. andraeanum*). *C. fructicola* (*C. gloeosporioides* complex) and *C. orchidearum* (*C. orchidearum* complex) were identified as pathogens causing the spathe rot by multigene DNA sequence analyses. Pathogenicity of the two species was confirmed.

The fungal pathogens associated with the spadix rot, or black nose, and leaf spot that are also manifested as anthracnose disease in anthurium, were identified as *C. fructicola* and *C. siamense* respectively.

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**Author's contribution** All authors contributed to the conception and design of the study. Material preparation, data collection

and analysis were performed by [I.S. Komala Vithanage], [D.M.D. Yakandawala], [S.S.N. Maharachchikumbura], [U.L.B. Jayasinghe] and [N.K.B. Adikaram]. The first draft of the manuscript was written by [I.S. Komala Vithanage & N.K.B. Adikaram] and all authors commented on subsequent versions of the manuscript. All authors read and approved the final manuscript.

**Declarations** The authors disclose that they have no conflict of interest.

No humans or animals were used to conduct the research work presented in this manuscript.

All the authors have given informed consent for submission of the manuscript.

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