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Identification and characterization of *Colletotrichum gloeosporioides* complex members from rubber plants in Sri Lanka

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Abstract

Colletotrichum leaf disease of rubber, caused by *Colletotrichum* species, can be considered one of the most severe diseases of *Hevea brasiliensis* in Southeast Asia. In Sri Lanka, both *C. gloeosporioides* and *C. acutatum* reported as causal agents of anthracnose on rubber trees. This paper presents a morphological, genetic, and pathogenic study conducted using *Colletotrichum* isolates belonging to the *C. gloeosporioides* species complex collected from different rubber cultivating areas of Sri Lanka. Here, except *C. gloeosporioides*, *C. siamense* has been recognized as the predominant species from *C. gloeosporioides* species complex causing rubber leaf disease in Sri Lanka. The identity has been confirmed using multilocus phylogeny analysis.

Keywords – Internal transcribed spacer region – Morphology – Phylogeny

Introduction

Hevea brasiliensis (Willd. ex Juss.) Muell. Arg. is the major source of natural rubber (NR) from the family Euphorbiaceae in the world (Thambugala & Deshapriya 2009). Rubber tree is subjected to many foliar diseases, causing yield reduction. Colletotrichum leaf disease of rubber is caused by *Colletotrichum* sp. is one of the most severe diseases of *Hevea brasiliensis* in Southeast Asia, including Sri Lanka, India, and China (Liu et al. 2018). *Colletotrichum* leaf disease of rubber is a major leaf disease that directly affect the latex production of rubber trees. The fungal pathogen causing *Colletotrichum* leaf disease in rubber develops three different disease symptoms: raised spots, anthracnose, and papery lesions (Saha et al. 2002). The pathogen was first identified as *C. heveae* and then assumed to be *Colletrotrichum gloeosporioides* (Liu et al. 2018).

Studies suggested that both *C. gloeosporioides* and *C. acutatum* are causal agents of anthracnose on rubber trees (Cao et al. 2018). According to Jayasinghe et al. (1997), the majority of

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strains that cause CLD in Sri Lankan rubber plantations belong to *C. acutatum*. Both *C. gloeosporioides* and *C. acutatum* are species complexes that contain species with unclear species boundaries. The *C. gloeosporioides* complex consists of 22 species and one sub species (Weir et al. 2012), while *C. acutatum* complex containing 31 species (Damm et al. 2012). However, recent studies have added new species into both these species complexes.

Species within a complex cannot be easily distinguished based on their morphological characteristics, such as colony color, conidial shape and size, and growth rate. In addition to those fungal morphological characters heavily rely upon the growth media, temperature, light, and host dependance (Lee et al. 2020). According, to Lee et al. (2020), some morphological characteristics, particularly colony color, may change or be lost with repeated subculturing. Therefore, molecular analysis is the most reliable approach in delineation of *Colletotrichum* species. Application of species-specific primers (White et al. 1990, Sreenivasaprasad et al. 1996), DNA fingerprints obtained using inter sequence simple repeats (ISSR) (Weining & Langridge 1991, Stenlid et al. 1994) that enable easy identification of pathogens with the help of presence or absence of specific bands and banding patterns and multilocus approach of phylogenetic analysis (Weir et al. 2012, Damm et al. 2012) are some widely applying techniques in *Colletotrichum* studies. The objective of this study was to identify, characterise, and test the pathogenicity of *Colletotrichum gloeosporioides* species complex causing Colletotrichum Leaf Disease of rubber in Sri Lanka.

Methodology

Sample collection and isolation of the pathogen

Infected twigs of Rubber were collected during June to December, 2012 from major rubber cultivating districts in Sri Lanka, including Kaluthara, Rathnapura, Colombo, Galle, Kegalle, Mathara and, Monaragala. After covering the cut end with wet cotton wool, detached twigs were placed in sterilized polypropylene bags. Collected samples were labeled according to the clone and locality prior to transport to the laboratory. A total of 165 infected rubber samples of 31 different rubber clones were collected from seven districts. Out of the total number of samples, causative organism was successfully isolated from 52 samples.

Infected sample area was surface sterilized using 70% ethanol and blot dried using sterilized tissue papers inside the laminar flow cabinet. Then the lesion was cut opened using a sterilized scalpel and placed on a fresh Potato Dextrose Agar (PDA) plate. Cultures were incubated at room temperature for up to four days. Subculturing of mycelia on new PDA plates was done on the consecutive 4^{th} day, to obtain pure cultures.

Morphological Characterization

Colors of upper and lower sides, nature of the margins, texture, appearance, shape and presence of concentric rings were recorded using 7– day old cultures on PDA. Five replicates from each sample were used for the experiment.

The growth rate of isolates on PDA was calculated by measuring the daily increment of the culture diameter for ten days. The cultures were prepared by placing a 7mm diameter mycelium disc obtained from a 7–day old culture of the isolate on a fresh PDA plate. Two diameter readings perpendicular to each other were taken at a time using five replicate cultures of each isolate. To determine the growth on liquid medium, the fungus was grown in 50 ml of Malt Extract broth in 250 ml Erlenmeyer flasks. Each broth was inoculated with a 7mm diameter mycelium disc obtained from a 7–day old culture of the fungus on PDA and incubated without shaking at 25^{o}C for 15 days. The mycelium was harvested at three-day intervals by filtering through Whatman No.1 filter paper. The harvested mycelium was oven-dried at 80^{o}C for 24 h and the weight was measured. The rate of increase in dry weight was taken as the rate of growth. At each point, three flasks were harvested. Slide cultures of the isolates on PDA were used to determine the color, shape, and dimensions of the conidia and appressoria. Randomly selected 100 conidia and 100 appressoria were used and the experiment was triplicated (Sutton 1980, Du et al. 2005). Conidia concentration in 7–day old PDA

cultures was measured by counting the number of conidia in a distilled water suspension using a counting chamber. To prepare the conidia suspension, 10 ml of distilled water was added to a 7–day old culture, gently shaken to suspend the conidia in water, and filtered through a muslin cloth to remove the mycelia. To measure the concentration of conidia in broth cultures, 7–day old broth culture prepared as described above was used. At the end of the seven days, flasks were gently shaken and filtered through a muslin cloth. The resulting filtrate was used to count the number of conidia using the counting chamber. Conidial concentration was calculated as the number of conidia produced per square centimeter of a seven-day-old culture. Shape, color, and distribution of conidial masses were also observed in the 7–day old culture on PDA using a stereomicroscope (Luxeo 2S).

Genetic Characterization

DNA was extracted from mycelia scraped from two-day-old cultures on PDA plates. All isolates were subjected to polymerase chain reaction (PCR) using ITS4 universal primer together with species-specific primer, CgInt for *C. gloeosporioides* (Sreenivasaprasad et al. 1996). Total volume of the PCR reaction mixture was 25 µl which included 1µl of 20-fold diluted genomic DNA; 50 mM KCL; 10 mM Tris-HCl; 0.2mM each dATP, dTTP, dGTP, dCTP; 1.5 mM $MgCl_2$; 2 units of Taq DNA Polymerase (UCBiotech, Sri Lanka), and 0.2 µM ITS4 primer and 0.2 µM CgInt primer. Genomic DNA of the reference cultures was added as the templates for the positive control and negative control. All the reactions were triplicated in a thermo cycler (Ependorf master cycler-USA) starting with 5 min of denaturation at 95^{o}C followed by 35 cycles of 30 s at 95^{o}C, 30 s at 60^{o}C, and 90 s at 72^{o}C (McKay et al. 2009) and 5 min of final extension at 72^{o}C. Amplified DNA products were separated using 1% (wt/vol) agarose gel incorporated with ethidium bromide (0.5µg/ml) in 1X Tris-acetate EDTA buffer (40 mM Tris acetate, 1mM EDTA, PH 8.3) by electrophoresis at 100v for 15 min. Observations were taken with the use of a gel documentation system. The isolates given positive results were selected to conduct multilocus phylogenetic study for the *Colletotrichum gloeosporioides* species complex. Isolates given negative results were tested for *C. acutatum* species complex (Hunupolagama et al. 2017)

Extracted genomic DNA samples of the positive isolates were stored in -20^{o}C and the cultures were preserved using mineral oil. Multilocus Phylogenetic study was conducted using the retrieved DNA from preserved samples in the year 2021.

Four nuclear gene regions, including internal transcribed spacer region (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), calmodulin (CAL), and β-tubulin 2 (TUB2) of 22 *Colletotrichum* isolates selected using species-specific primers for *C. gloeosporioides* were amplified using primer pairs, ITS1Ext and ITS4Ext, GDF and GDR, CL1C and CL2C, T1 and Bt2b respectively. In amplification of ITS region, each PCR reaction (25 µl) contained 20 ng of genomic DNA, dNTP (0.2 mM each), forward and reverse primer (0.2 µM), $MgCl_2$ (2 mM), PCR buffer (50 mM KCl, 10 mM Tris – HCl, pH 9.0) and 2 units of Taq DNA polymerase. For the other three regions, the concentration of the PCR buffer, Taq DNA polymerase was the same as above with 1.5 µl of DMSO. PCR protocol for the ITS region was started with 5 mins initial denaturation at 95℃ followed by 35 cycles of the 30s at 95℃, 30 s at 60℃, and 90s at 72℃. The final extension was 5 mins at 72℃. PCR protocol for GAPDH, TUB2, and CAL regions was started with 5 mins initial denaturation at 94℃ followed by 35 cycles of the 30s at 95℃, 30s at 60℃ for GAPDH, 55℃ for TUB2 and 58℃ for CAL and 45s at 72℃. The final extension was 7 mins at 72℃. Primers were used to amplify the four gene regions. Forward and the reverse primer of the ITS region were ITS1Ext and ITS4Ext respectively (Talhinhas et al. 2002). For the GAPDH gene, GDF was selected as the forward primer while using GDR as the reverse primer (Templeton et al. 1992). The forward primer of the CAL gene was CL1C and the reverse primer was CL2C (Weir et al. 2012). For TUB2 gene T1 and Bt2b were forward and reverse primers respectively (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997).

Pathogenic Testing

Conidial suspensions with 1 x 10^6 concentration were prepared from well sporulated pure cultures of *C. gloeosporioides* and *C. siamense* (Tshering 2006). Matured healthy rubber twigs containing 4 to 5 leaves from each test rubber clone were surface sterilized (Sanders & Korsten 2003). The upper sides of the rubber leaves of each clone were spray-inoculated using the prepared conidial suspensions. Sterilized distilled water was used for controls. Inoculated samples and controls were incubated for 5 to 10 days at 25^{o}C in moisture chambers with 95% relative humidity and the cut ends were placed in sterilized distilled water throughout the experimental period (Than et al. 2008), and number of lesions raised in each sample was counted. Full experiment was replicated in five times.

Data Analysis

Resulted sequences were subjected to initial identification using BLASTN search option of NCBI database. Then ITS, GAPDH, ACT and TUB2 sequences obtained from all isolates and type sequences downloaded from Genbank was aligned using Muscle (Edgar & Robert 2004). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura & Nei 1993). The tree with the highest log likelihood (-10638.44) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 2793 positions in the final dataset. Evolutionary analyses were conducted in MEGA11. The analysis involved concatenated nucleotide sequences of 45 strains, including type sequences of species belonging to the *C. gloeosporioides* species complex and of *C. orchidophilum* as out-group (Fig. 4). Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. Bootstrap analysis was conducted with 1000 random additions.

Results & Discussion

Morphological Characterization

Arial mycelium of all *C. gloeosporioides* cultures was greyish white and cottony. The reverse side of the cultures varied in color from off-white to light greenish-grey with an orange center. Orange, sessile conidial masses were observed mainly at the center of the culture. Conidia generating cells were clavate or cylindrical. Conidia were hyaline, cylindric to clavate with rounded ends or slightly tapering from one end. Grey to light brown, dark walled appressoria were clavate, oval, or irregular shaped and variable in size (Fig. 1) (Table 1).

Arial mycelia of all *C. siamense* cultures was grey and cottony. The reverse side of the cultures was off-white, orange, and dark green. Conidiogenous cells in branched conidiophores were oblong or cylindrical. Conidia were hyaline, cylindrical with rounded ends or slightly tapering from one end. Dark grey colored appressoria with a dark wall were oval or irregular shaped and variable in size (Fig. 2) (Table 1).

Genetic Characterization

Twenty-five *Colletotrichum* isolates from rubber plants amplified a 420 bp PCR fragment with *C. gloeosporioides* specific primer, CgInt. In the phylogenetic tree generated from Maximum Likelihood analysis of the combined data set of ITS, GADPH, TUB2 and CAL gene regions, isolates of *C. gloeosporioides* obtained in this study and the type culture formed a distinct clade with 100% bootstrap support. *C. siamense* isolates and type culture were also well separated with 98% bootstrap support.

Colletotrichum isolates have been successfully identified up to the species complex and species levels. Twenty-five isolates that provided positive results with the primer CgInt, belonged

to the *C. gloeosporioides* species complex and 22 of them represent two species including *C. gloeosporioides* and *C. siamense.* The remaining isolates could not be identified by multilocus based phylogenetic analysis due to the absence of some sequences.

Pathogenic Testing

Fulfilling the Koch's postulates, rubber leaves inoculated with *C. gloeosporioides* and *C. siamense* started to express disease symptoms. Rubber leaves exhibited brown to black color necrotic spots on the leaf surface.

C. gloeosporioides and *C. acutatum* have been recorded as the causative agents of rubber leaf disease in Sri Lanka (Jayasinghe et al. 1997, Jayasinghe & Fernando 1998). Among them *C. gloeosporioides* belongs to *C. gloeosporioides* species complex. In addition to *C. gloeosporioides* species, *C. siamense* has been recognized as the predominant species from *C. gloeosporioides* species complex causing rubber leaf disease in Sri Lanka.

Even shapes of conidia varied from species to species it is possible to identify the most abundant shapes of conidia for each species. However, a pattern could not be identified. As for conidia, appressoria with various shapes and sizes was identified. Generally, the members of the *C. gloeosporioides* complex have a relatively high growth rate as well as radial growth. These findings are agreed with observations reported by Damm et al. (2012), Weir et al. (2012), and Liu et al. (2014).

All isolates of rubber plant from *C. gloeosporioides* complex were amplified a 420 bp PCR fragment with *C. gloeosporioides* specific primer, CgInt, representing two species including *C. gloeosporioides* and *C. siamense.* species specific primers for *C. gloeosporioides* (CgInt and ITS4) can be used to identify members of *C. gloeosporioides* complex (Sreenivasaprasad et al. 1996).

Maximum Likelihood tree generated using combined sequences of ITS, GADPH, CAL and TUB2 gene regions of isolates belonging to the *C. gloeosporioides* species complex with the same combined sequences of reference isolates confirmed the identity of *C. gloeosporioides* and *C. siamense*.

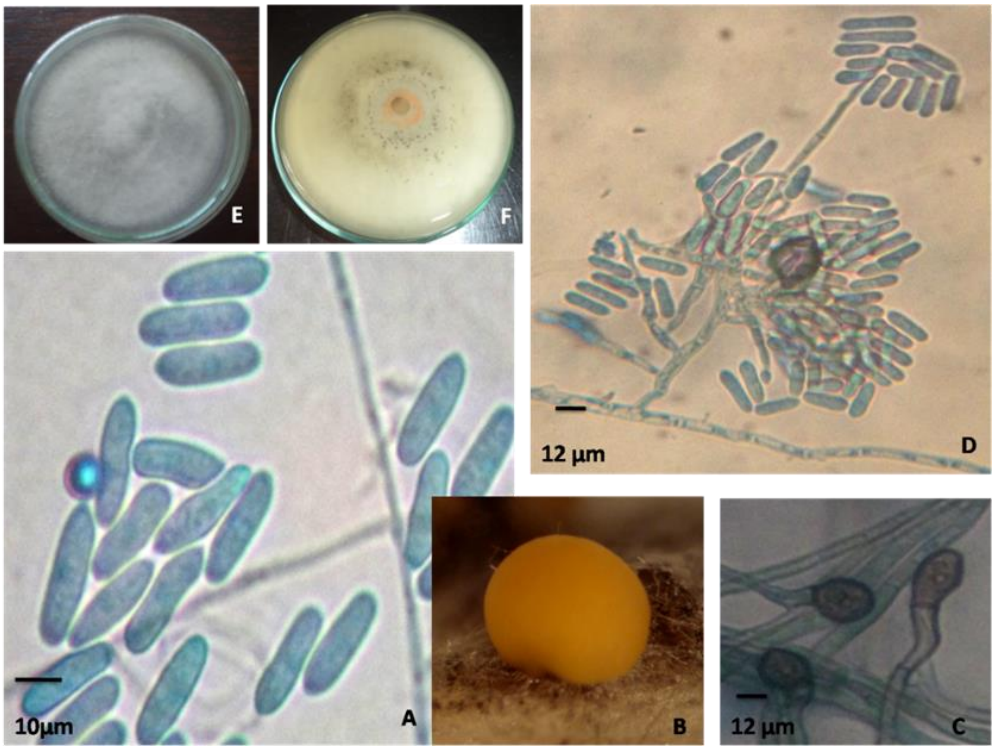

Fig. 1 – *Colletotrichum gloeosporioides*. A Mature conidia. B Conidial mass. C Appressoria. D Conidiophore bearing conidia and conidiogenous cells. E Aerial mycelium. F Reverse side of a

10–day old culture on PDA. Conidia, appressoria and conidiophores were stained using Cotton blue reagent.

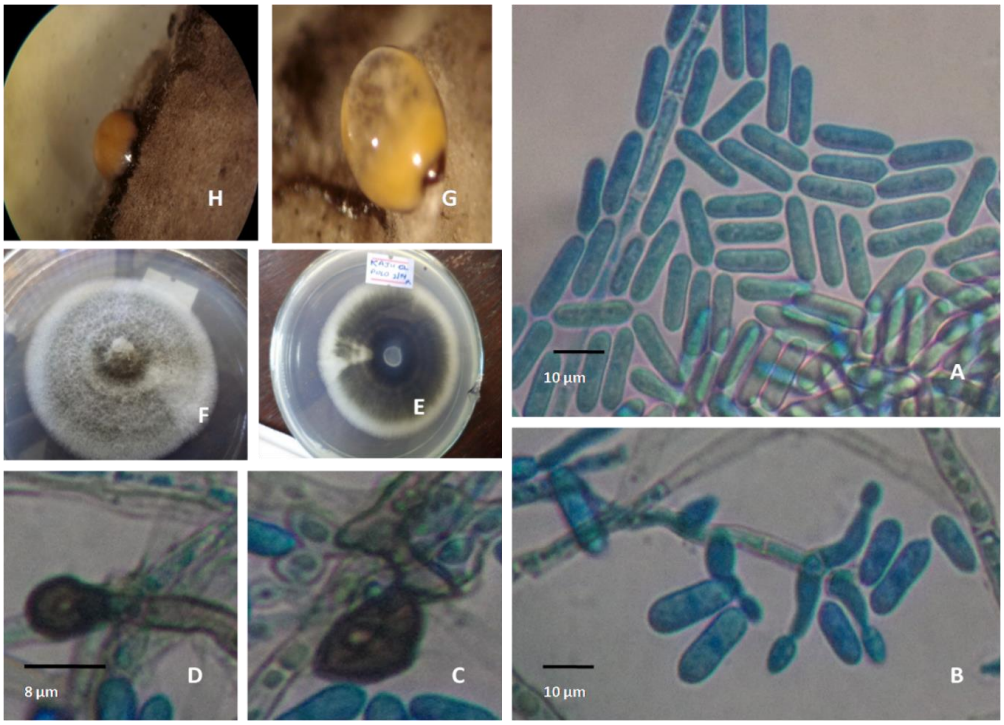

Fig. 2 – *Colletotrichum siamense*. A Mature conidia. B Conidiophore with conidiogenous cells. C, D Appressoria. E Reverse side. F Aerial mycelium of a ten days old PDA culture. G, H Conidial masses.

Table 1 Morphological characters of different isolates of *Colletotrichum gloeosporioides* complex isolated from diseased rubber samples.

Name	Conidia		Appresoria		Growth rate	
	Mean length (µm)	**Mean width (µm)**	**Mean length (µm)**	**Mean width (µm)**	**Radial growth rate (mm/day)**	**Rate of increase of dry weight (g/day)**
C. gloeosporioides	15–30–33.4	5–7.5–8.5	9.3–15–17.1	7–7.5– 10.1	10	2.25
C. siamense	12–20.115.3	4.5–7.5-8.5	9.3–22.525.1	7 – 15 – 18.1	8	2.25

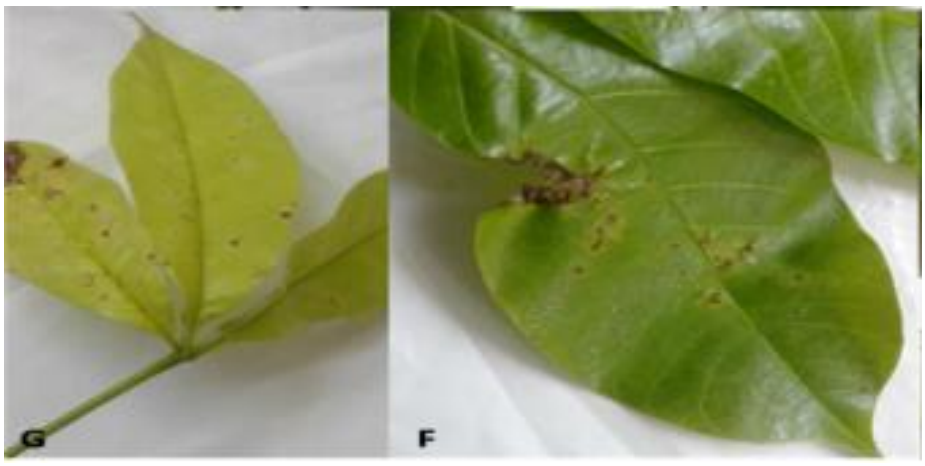

Fig. 3 – F -RRISL 260 inoculated with *C. gloeosporioides* and G -RRISL 212 inoculated with *C. siamense*.

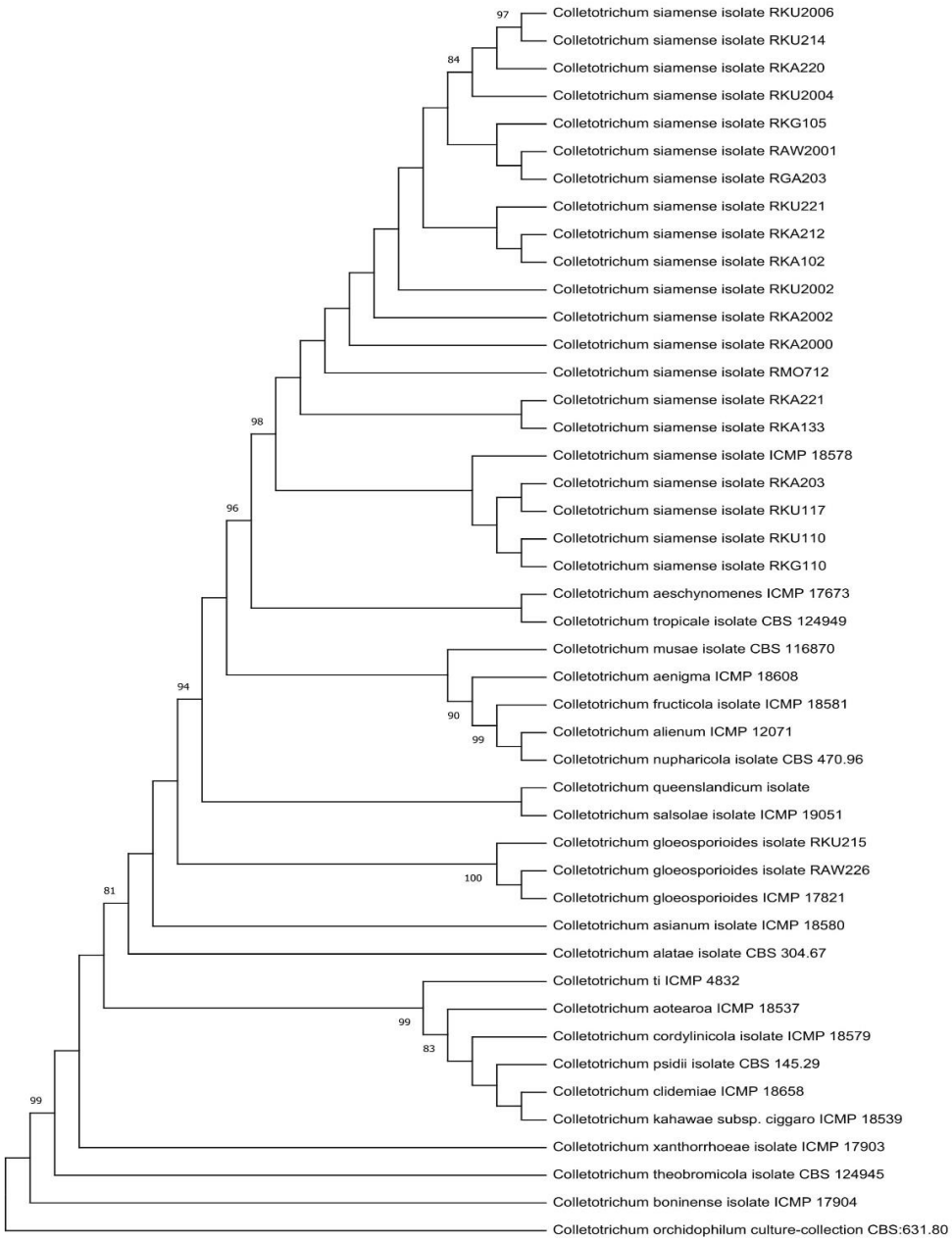

Fig. 4 – Phylogenetic tree generated from maximum likelihood analysis of the combined data set of ITS, GAPDH, TUB2 and CAL genes of *Colletotrichum* isolates from Sri Lankan rubber and type sequences belonging to the *C. gloeosporioides* species complex. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together in the bootstrap analysis (1000 bootstraps) is shown next to the branches.

Conclusions

The overall result of this investigation establishes the fact that *C. gloeosporioides* is not the only species from the *C. gloeosporioides* complex causing rubber anthracnose in Sri Lanka. Other than that *C. siamense* was identified as the major causative agent of rubber anthracnose from the same species complex, and a higher percentage of the isolates was reported from the same. Like in other countries, there can be more different species from *C. gloeosporioides* species complex causing rubber anthracnose in Sri Lanka. A study with a larger number of isolates can be recommended for further investigation of species diversity.

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