Unveiling Members of Colletotrichum acutatum *Species Complex Causing* Colletotrichum *Leaf Disease of* Hevea brasiliensis *in Sri Lanka*

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Unveiling Members of *Colletotrichum acutatum* Species Complex Causing *Colletotrichum* Leaf Disease of *Hevea brasiliensis* in Sri Lanka

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Abstract *Colletotrichum* is an important fungal genus with great diversity, which causes anthracnose of a variety of crop plants including rubber trees. *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* have been identified as the major causative agents of *Colletotrichum* leaf disease of rubber trees in Sri Lanka based on morphology, pathogenicity, and the analysis of internally transcribed spacer sequences of the nuclear ribosomal DNA. This study has been conducted to investigate the members of the *C. acutatum* species complex causing rubber leaf disease using a morphological and multi gene approach. For the first time in Sri Lanka, *Colletotrichum simmondsii, Colletotrichum laticiphilum, Colletotrichum nymphaeae*, and *Colletotrichum citri* have been identified as causative agents of *Colletotrichum* leaf disease in addition to *C. acutatum* s. str. Among them, *C. simmondsii* has been recognized as the major causative agent.

Keywords Sri Lanka · *Colletotrichum* · Rubber · Anthracnose

Introduction

Colletotrichum is a wide-spread fungal genus containing many pathogenic species. These species affect almost all parts of a variety of plants, including vegetables, cereals, fruits, and legumes [1, 3, 20, 29] distributed around the world. Among them, *Colletotrichum* leaf disease (CLD) is considered as a major disease of rubber trees (*Hevea brasiliensis*) in Southeast Asian countries including Sri Lanka [22, 30]. CLD of rubber trees, mainly causes significant reduction of rubber yield by secondary leaf fall [22, 25, 41]. Small circular or large lesions on mature and immature leaves can be identified as a common disease symptom. In epidemics, it also affects young twigs with premature leaves leading to blackening of tips and brown to black circular or oval typical anthracnose lesions on green stems.

Most *Colletotrichum* species isolated from rubber trees in different countries belong to *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* species complexes [2, 7, 12, 15, 32, 45, 46]. In addition, *Colletotrichum crassipes* [41], *Colletotrichum dematium* s. lat [44], and *Colletotrichum annellatum*, the latter belonging to *Colletotrichum boninense* complex [8] have also been isolated from rubber trees. In Sri Lanka, *C. gloeosporioides* s. lat. and *C. acutatum* s.lat. were previously identified as the causative agents of CLD of rubber trees [21, 22]. They can act individually or synergistically with each other to cause the disease by forming larger lesions [40]. Furthermore, according to the available records in Sri Lanka, *C. acutatum* is the major causative agent of CLD of rubber trees [22]. However, the identification of *C. acutatum* causing

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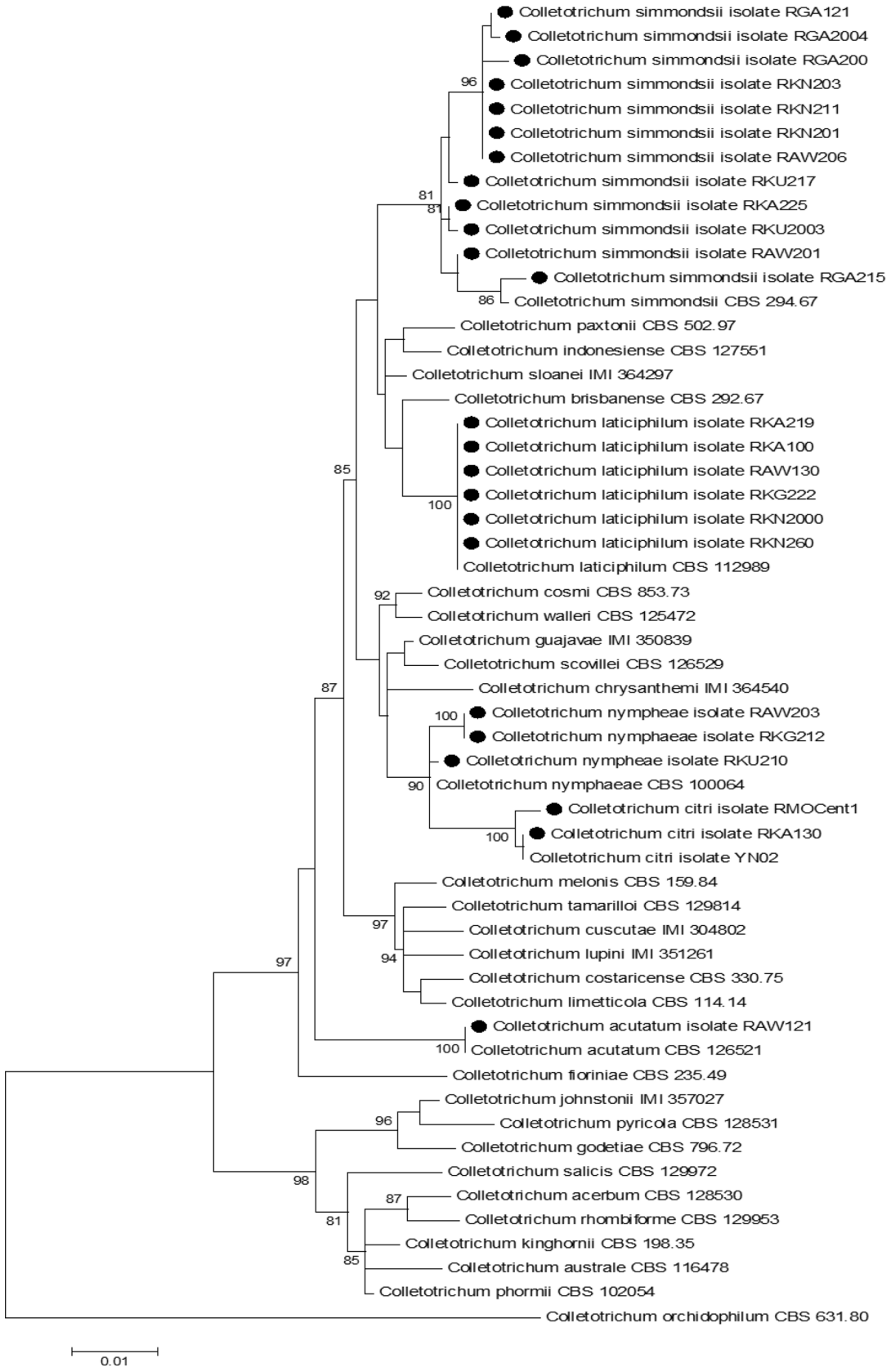

◂**Fig. 1** Phylogenetic tree generated from maximum likelihood analysis of the combined dataset of ITS, GAPDH, and TUB2 genes of *Colletotrichum* isolates belonging to the *C. acutatum* species complex from rubber trees. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together (bootstrap value) is shown next to the branches. The tree was rooted using *C. orchidophilum* as out-group. *Colletotrichum* species isolated in this research are marked with *black bullets*

CLD of rubber trees in Sri Lanka has been mainly based on the morphology of conidia and cultures, growth rates, or fungicide resistance [21]. To date, no molecular data have been used for its identification or characterization. Furthermore, phylogenetic positions of these isolates have not been determined with reference to the other isolates in the same species and different species of *Colletotrichum* reported worldwide. For more than a decade, no research has been conducted to investigate the diversity of these pathogens in Sri Lanka.

Within the past 15 years, many morphological and molecular techniques have been used to resolve the phylogenetic positions of different *Colletotrichum* species including *C. acutatum*. Initially, use of the internal transcribed spacer (ITS) regions was considered the best method to resolve the species boundaries [4, 35, 36]. Later different protein coding gene regions like beta-tubulin, calmodulin, glyceraldehyde-3-phosphate dehydrogenase, chitin synthase 1, and histone 3 were also identified [5, 6, 13, 27, 28, 39] as suitable regions for fungal characterization. Finally, different combinations of these gene regions were recommended [7, 11, 14, 31, 33] to resolve the *Colletotrichum* species and separate them into species complexes.

With the identification of the *C. acutatum* species complex [7] and the description of novel species within that complex and due to the absence of recent studies on that complex in Sri Lanka, this research has been conducted using morphological characters together with a multi-gene approach to determine the diversity and the phylogeny of members of the *C. acutatum* species complex on rubber trees in Sri Lanka with reference to the published records worldwide.

Materials and Methods

Sample Collection, Isolation, DNA Extraction, and Initial Identification

Diseased plant materials belonging to different clones of rubber trees were collected from the seven major rubber cultivating districts in Sri Lanka (Online resource 1) from September 2012 to August 2014. Shoots and twigs containing immature and mature leaves with black to brown colored necrotic lesions on the leaf surface were detached from the plants and placed in sterilized polypropylene bags after covering the cutting end with wet cotton wool. After labeling, the samples were transported to the laboratory. Infected leaves were surface sterilized using 70% ethanol then dried using sterilized tissue paper inside a laminar flow cabinet. Small pieces of diseased leaf tissues were separated using a sterilized scalpel and placed on a fresh potato dextrose agar (PDA) plate. The inoculated plates were incubated at 27 °C. Then the mycelium arising from the diseased tissue was sub-cultured on a new PDA plate to obtain a pure culture. Finally, single conidia cultures were prepared [17] for further experiments.

Genomic DNA was extracted from all isolates using a protocol optimized previously [18]. The 5.8S nuclear ribosomal gene with the flanking internal transcribed spacer regions was amplified using ITS1EXT and ITS4EXT primers [26, 28] for initial identification of members of the *C. acutatum* complex. PCR reactions were performed using a thermo cycler (Eppendorf master cycler, USA) with 5 min of denaturation at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C and 90 s at 72 °C [24] and 5 min of the final extension at 72 °C. PCR products were separated using gel electrophoresis and then bidirectionally sequenced. The sequences were analyzed using BioEdit version 7.2.0 [16]. A blastn search in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with all ITS sequences was conducted for initial identification of the isolates; isolates showing more than 90% similarity to members of the *C. acutatum* species complex were selected for further analysis (Online Resource 1).

Phylogenetic Study

In addition to the ITS region, two nuclear gene regions were used for the phylogenetic study of the selected isolates belonging to the *C. acutatum* complex. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-tubulin 2 (TUB2) were amplified using the primer pairs, GDF and GDR [39], T1 [27] and Bt2b [13], respectively. For amplification of the two gene regions, 2 mM $MgCl_2$ and 1.5 µl DMSO were used; the quantities of the PCR buffer, genomic DNA, and Taq DNA polymerase were the same as those of the amplification of ITS. PCR was carried out with a 5 min initial denaturation at 94 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C for GADPH, 55 °C for TUB2, and 45 s at 72 °C. The final extension was 7 min at 72 °C. The resulting PCR products were sequenced and all sequences were deposited to GenBank (Online Resource 1). Sequences of the three gene regions were combined with each other to obtain concatenated sequences for each isolate and then aligned using Muscle [10].

Table 1 Morphological characters of 7-day-old PDA cultures of *C. acutatum, C. simmondsii, C. laticiphilum, C. nymphaeae*, and *C. citri* isolated in this study

Species	Colony color of a 7 days old culture on PDA		Conidia	Appressoria	Radial growth rate (cm/day)	
	Upper side	Reverse side				
C. acutatum	Gray	Orange or white	18 µm × 4 µm Oblong with pointed ends	7.5 µm × 6 µm Brown color, oblong or ovate	0.54	Figure 2
C. simmondsii	White to gray	Salmon pink, green or white	16.5 µm × 5 µm Fusiform	6 µm × 5.5 µm Gray color, oblong or ovate	0.49	Figure 3
C. laticiphilum	White with brown middle	Brownish green or offwhite	17.5 µm × 5.5 µm Oblong with one end pointed or both ends rounded	10.5 µm × 8.5 µm Gray color, oblong or ovate	0.57	Figure 4
C. nymphaeae	White	Pink or white	17.6 µm × 4.5 µm Oblong with one end pointed or both ends rounded	9 µm × 7.5 µm Gray color, oblong or ovate	0.53	Figure 5
C. citri	White	Offwhite	17 µm × 4.5 µm Oblong with one end pointed or fusiform	10 µm × 7.5 µm Gray color, polygonal or ovate	0.68	Figure 6

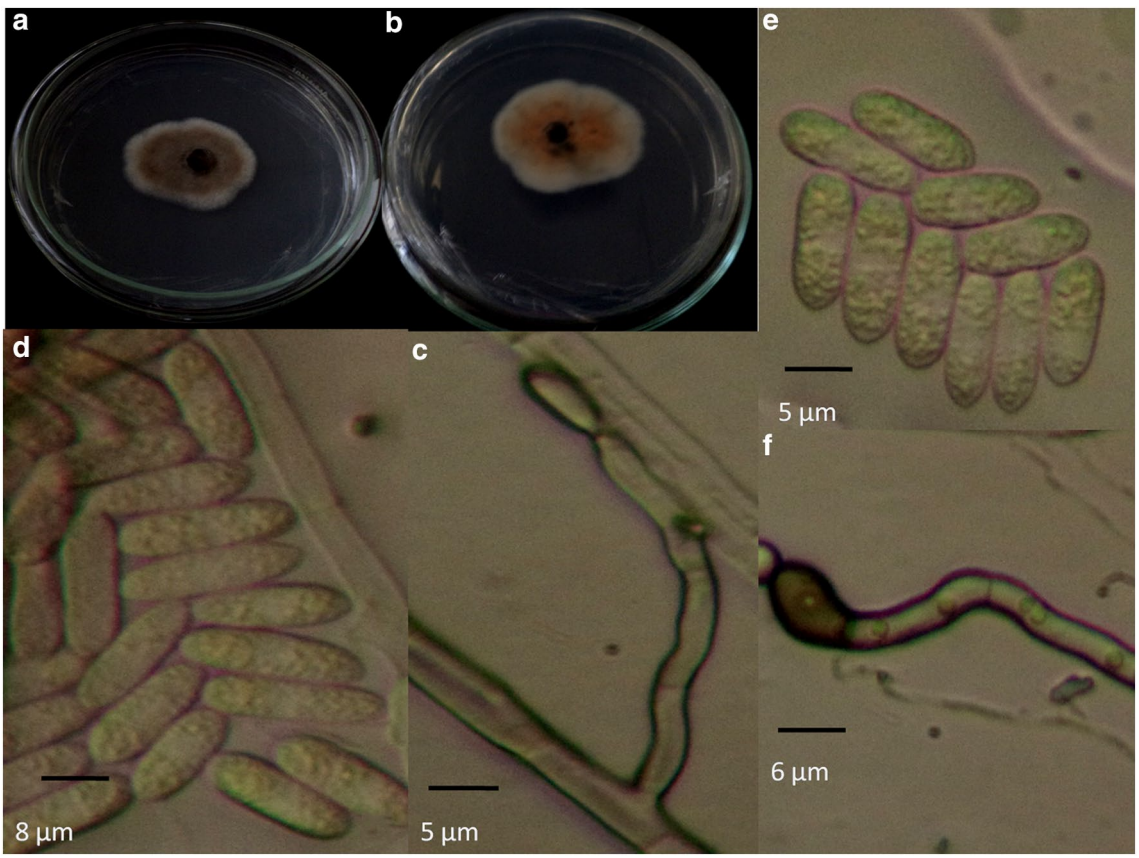

Fig. 2 *Colletotrichum acutatum* (RAW121). **a** Upper side and **b** reverse side of a 10-day-old culture on PDA, **c** Conidiophore, **d** and **e** Conidia, **f** Appressorium

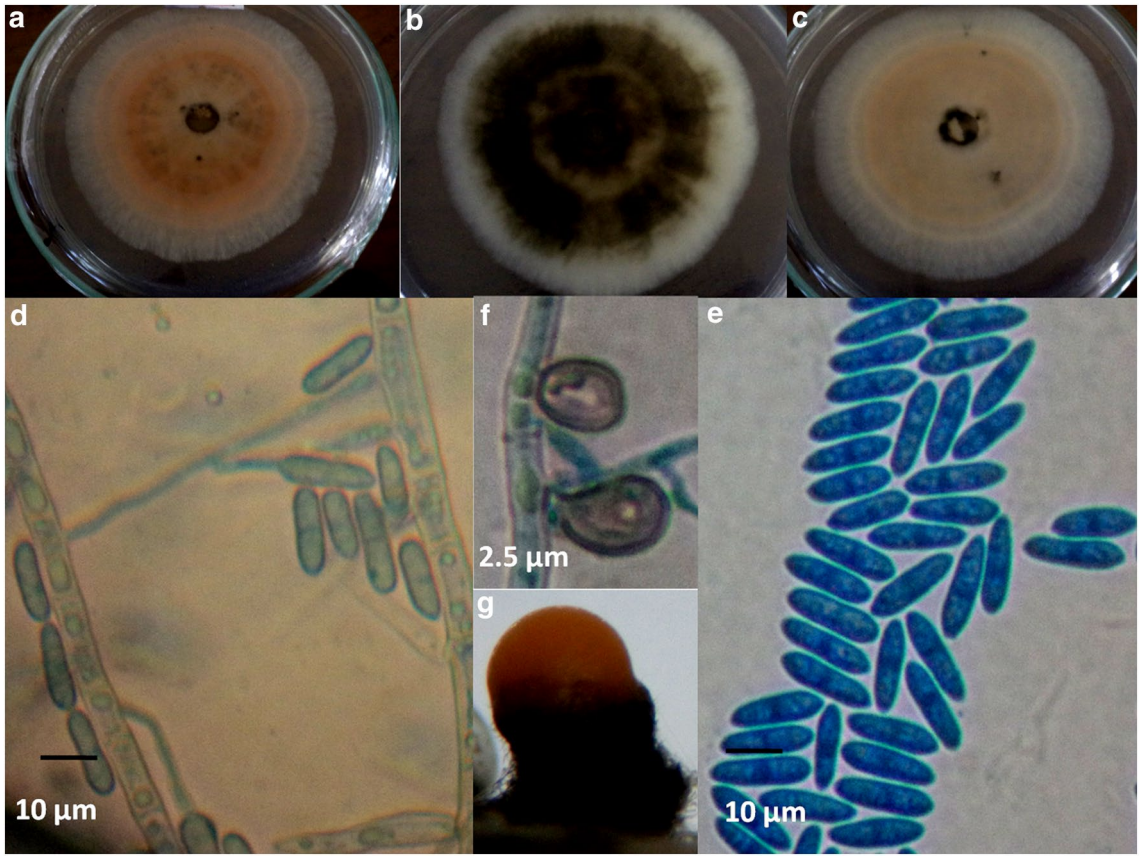

Fig. 3 *Colletotrichum simmondsii* (RGA200). **a–c** Reverse side of three types of 10-day-old PDA cultures, **d** Conidiophore, **e** Conidia, **f** Appressoria, **g** Conidial mass developing from a conidiomata

The phylogenetic relationships were inferred using the maximum likelihood (ML) method based on the Tamura three-parameter model [37]. Initial tree(s) for the heuristic search were obtained automatically, by applying neighbor joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter = 0.1000)]. The tree is drawn to scale, with branch lengths measured by the number of substitutions per site. The analysis involved concatenated nucleotide sequences of 55 strains, including reference sequences of all species belonging to the *C. acutatum* species complex, sequences of the *Colletotrichum* strains isolated during this study and of *Colletotrichum orchidophilum* as out-group. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 688 positions in the final dataset. Analyses were carried out using MEGA6 [38]. Bootstrap analysis was conducted with 1000 random additions.

Morphological Study

The color of the upper and lower sides of the cultures, radial growth rate, production of conidial masses, and presence or absence of concentric rings were observed and recorded using 7-day old single conidia cultures on PDA. Each culture had five replicates. The radial growth rate was calculated by averaging the daily increment of colony diameter throughout a 10-day period. Using 7-day-old slide cultures, dimension of the conidia and appressoria was measured [9, 34]. Three slide cultures were prepared from each isolate. 100 conidia and 100 appresoria were studied from each slide culture.

Pathogenicity Test

Ten milliliters of sterilized distilled water were added to 14-day-old well sporulating single conidia cultures of *C. acutatum* (RAW121), *Colletotrichum simmondsii* (RKN211), *Colletotrichum nymphaeae* (RKU210), *Colletotrichum laticiphilum* (RKA100), and *Colletotrichum citri* (RMOCent1) on PDA. Plates were then gently swirled to

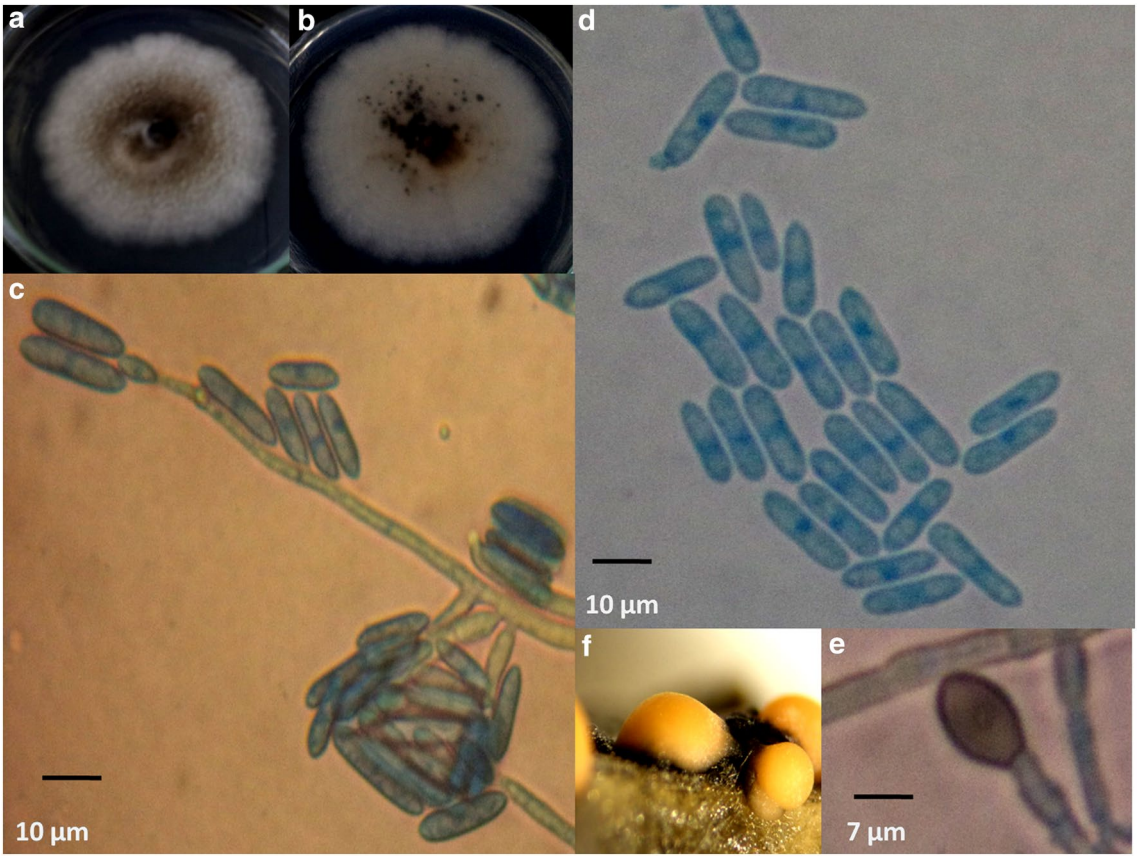

Fig. 4 *Colletotrichum laticiphilum* (RKA100). **a** Upper side and **b** reverse side of a 10-day-old culture on PDA, **c** Conidiophore, **d** Conidia, **e** Appressorium, **f** Conidial masses developing from conidiomata

mix well. The resulting conidial suspensions were filtered through two layers of sterilized muslin cloth each to remove mycelia, and the concentration of conidia was adjusted to 1×10^6/ml using a hemocytometer [42].

Mature healthy twigs containing 4–5 leaves of the test rubber clones were first washed with running tap water for 1 min followed by spraying with 70% ethanol. The samples were then immediately washed three times with sterilized distilled water and dried with sterile tissue papers. The upper sides of the leaves were spray inoculated using the prepared conidial suspensions. Sterilized distilled water was used in place of conidia suspension for the preparation of controls. Inoculated samples and controls were covered by punched polyethylene bags and cut ends were placed in sterile tap water throughout the experiment. They were then incubated for 5–10 days at 25 °C. Relative humidity was maintained at around 95%, by placing sterilized cotton balls soaked in sterilized distilled water inside the polyethylene bags [41]. On the seventh day, the diameter of the lesions and the number of lesions on each sample was counted. Then the pathogens were re-isolated from the lesions and the morphology of the resulting cultures was compared with the original cultures used for inoculation.

Results

After examination of the 98 infected rubber leaf samples, 52 were identified as infected by *Colletotrichum* spp. Based on the similarity of more than 90% of the ITS sequences to members of the *C. acutatum* species complex, 24 isolates were selected for further experiments.

The phylogenetic tree generated from maximum likelihood analysis of the combined sequences of ITS, GAPDH, and TUB2 genes, the studied isolates were clearly separated into five species; *C. acutatum, C. simmondsii, C. laticiphilum, C. nymphaeae*, and *C. citri* (Fig. 1) by clustering them with the reference isolates. The tree with the highest log likelihood (−1287.7317) was shown. The percentage of trees in which the associated taxa clustered together was shown next to the branches.

The 12 *C. simmondsii* isolates and the reference isolate CBS 294.67 clustered together with 81% bootstrap support. According to this study, six *C. laticiphilum* isolates and the reference isolate CBS 112989 clustered with 100% bootstrap support. Further, the clades of *C. acutatum* and *C. citri* isolates including their reference isolates also were well supported by bootstrap values of 100%. *C. nymphaeae*

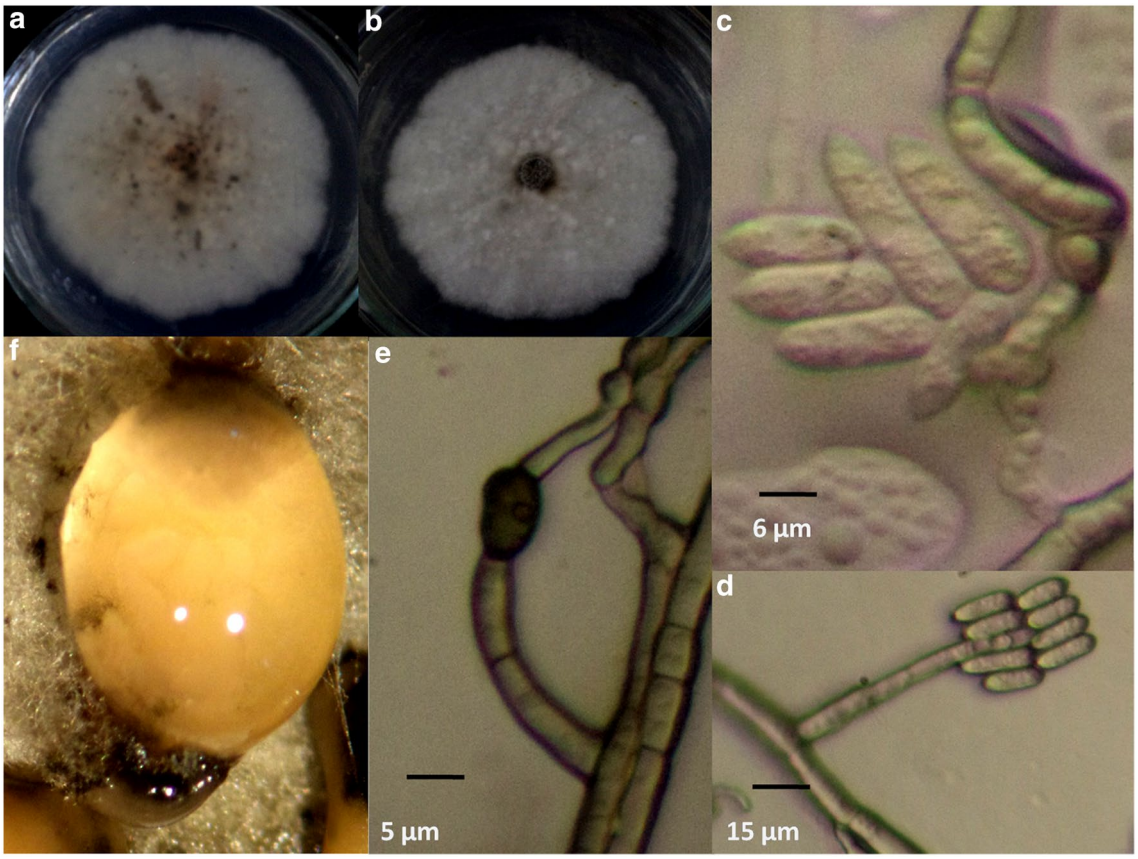

Fig. 5 *Colletotrichum nymphaeae* (RKG212). **a** Reverse side and **b** upper side of a 10-day-old culture on PDA, **c** Conidia, **d** Conidiophore, **e** Appressorium, **f** Conidial mass developing from a conidiomata

isolates also clustered together with the reference isolate, the bootstrap value was 90%. This result confirms the identity of 24 *Colletotrichum* isolates.

Morphological characters observed in the 7-day-old PDA cultures of the five species are described in Table 1. Colors of the upper side and the lower side of the cultures, conidia and appressoria dimensions, shapes, and colors were compared with the characters recently recorded by the researchers in other countries [7, 19]. Except *C. acutatum*, all species produced conidial masses after 5–10 days. Concentric rings were present in the cultures of all species. *Colletotrichum citri* was the fastest growing species compared to the others while *C. simmondsii* was the slowest growing species. Figures 2, 3, 4, 5, and 6 represent photographs of the cultures, conidia, appressoria, conidial masses, and conidiophores of each species.

After 5 days of inoculation, all the *Colletotrichum* species identified in this study developed disease symptoms on the rubber leaves of the respective rubber clones. Although the symptoms were the same, the lesion sizes produced by each species were smaller than the lesions on the original samples. Further, *C. nymphaeae* and *C. citri* produced higher numbers of lesions with a small diameter (<3 mm) while the other three species produced low numbers of lesions with large diameters (4 < 6 > 9 mm). However, *C. nymphaeae* was the most virulent species which produced the highest number of lesions, covering a higher percentage of the leaf area than the other species. Pathogens were successfully re-isolated from the lesions, confirming Koch's postulates, the morphology of the resulting cultures being similar to the original cultures.

Discussion

CLD directly affects the latex production of rubber trees by reducing leaf area which is used for photosynthesis. Since rubber latex is the product which matters economically, controlling this disease has become essential. Findings of our research will help to identify the *Colletotrichum* species responsible for CLD in Sri Lanka, their distribution as well as controlling them effectively. Prior to our study, *C. acutatum* was the only species of the *C. acutatum* complex identified as a causal agent of CLD of rubber trees in Sri Lanka. With the results of this study, four new members of the *C. acutatum* complex, *C. simmondsii, C. laticiphilum,*

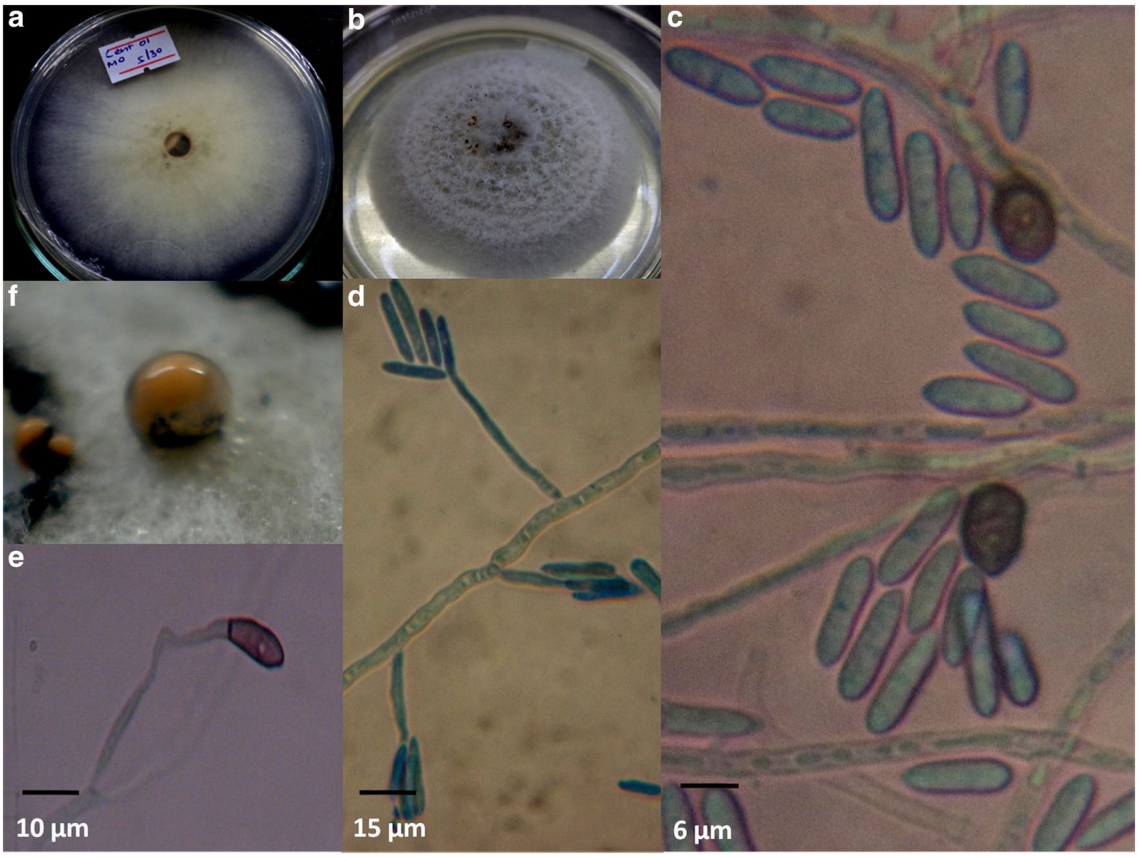

Fig. 6 *Colletotrichum citri* (RMOCent1). **a** Reverse side and **b** upper side of a 10-day-old culture on PDA, **c** Conidia and appressoria, **d** Conidiophores, **e** Appressorium, **f** Conidial mass developing from a conidiomata

C. nymphaeae, and *C. citri* have been identified as causal organisms of CLD of rubber, in addition to *C. acutatum*. Damm et al. established the term *C. acutatum* complex in 2012 and the four species, which we identified, were recently recognized as part of that complex. These newly recorded species may have been previously identified as *C. acutatum*.

Among the five species, *C. simmondsii* and *C. laticiphilum* showed a greater geographical distribution than did the other species. They were both isolated from six districts, including Kurunegala, Colombo, Ratnapura, Kalutara, Galle, and Kegalle. *Colletotrichum nymphaeae* was isolated from three districts; Ratnapura, Colombo, and Kegalle, while *C. citri* was isolated from the Monaragala and Kalutara districts. Unlike the other species, *C. acutatum* was isolated only from the Colombo district. However, from the 24 *Colletotrichum* isolates, 12 were *C. simmondsii* isolates. Hence, *C. simmondsii* can be regarded as the major causal agent of CLD of rubber trees in Sri Lanka belonging to the *C. acutatum* complex. When considering the species diversity, all species were recorded in the Colombo district except for *C. citri*. However, from the Monaragala and Galle districts only *C. simmondsii* and *C. citri*, respectively, were recorded in our study. These distribution details will be useful for taking effective measures to control the CLD of rubber trees.

Colletotrichum laticiphilum was described by Damm et al. [7] and they studied the infected rubber leaf samples collected from India and Colombia. According to them, this species was earlier identified by different names by different authors but exclusively isolated from rubber plants. In our study, we isolated *C. laticiphilum* from rubber plants representing six rubber clones (RRISL219, RRISL100, RRISL130, RRISL222, RRISL2000, and RRISL260). None of the other isolated *Colletotrichum* species is host specific. *Colletotrichum nymphaeae* was previously isolated from different crops such as apple, water-lilies, and black locust as a pathogen [23, 43, 47]. *Colletotrichum citri* has been reported from cultivated citrus in China [19]. However, we did not find any record of the above two species causing anthracnose on rubber plants. On the other hand, *C. acutatum* is a well-known fruit rot pathogen and is also able to infect all parts of the host plants. It was recorded in many fruits and vegetables. *Colletotrichum simmondsii* has been reported from many crops including

mango, avocado, and capsicum. Both of the above species also have been reported as rubber plant pathogens [22, 33].

All species we identified in this research were pathogenic to rubber trees in Sri Lanka. Among them, *C. nymphaeae* was the most aggressive pathogen which covered highest percentage of the rubber leaf area by forming circular necrotic lesions. Finally, this study has shown for the first time in Sri Lanka that *C. simmondsii, C. nymphaeae, C. laticiphilum*, and *C. citri* belonging to *C. acutatum* complex in addition to the *C. acutatum* are the causative organisms of *Colletotrichum* leaf disease of rubber trees. Among them, *C. simmondsii* can be identified as the major causative agent, which has a higher geographical distribution and *C. nymphaeae* as the most pathogenic species in *C. acutatum* complex causing the CLD of rubber in Sri Lanka.

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