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# *Colletotrichum* species associated with cultivated citrus in China

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**Abstract** There have been considerable advances in the understanding of species concepts in the genus *Colletotrichum*. This has lead to the need to carry out fresh surveys of *Colletotrichum* species associated with important hosts. *Colletotrichum* species are associated with *Citrus* plants as saprobes, important pre-harvest and post-harvest pathogens, as well as endophytes. In this study, a total of 312 *Colletotrichum* strains were isolated from leaves, shoots and fruits of cultivated *Citrus* and *Fortunella* species with or without disease symptoms across the main citrus production areas in China. The morphology of all strains were studied and multilocus (ACT, TUB2, CAL, GAPDH, GS, ITS) phylogeny established. Strains were from four important species complexes of *Colletotrichum*, namely *C. gloeosporioides* species complex, *C. boninense* species complex, *C. acutatum* species complex and a final group including *C. truncatum*, which was rare on *Citrus* species. The species belonging to the *C. gloeosporioides* species complex comprised *C. gloeos porioides* and *C. fructicola*, the *C. boninense* complex comprised *C. karstii* and a new species *C. citricola* and the *C. acutatum* complex included a new species, *C. citri*. The ability of strains to cause anthracnose on citrus fruits was tested by inoculation and strains of *Colletotrichum gloeosporioides*, *C. fructicola* and *C. truncatum* were pathogenic.

**Keywords** Citrus industry · Citrus diseases · Anthracnose · *Colletotrichum gloeosporioides* · *Colletotrichum acutatum* · *Colletotrichum boninense* · Morphology · Phylogenetic analysis

**Electronic supplementary material** The online version of this article (doi:10.1007/s13225-013-0232-y) contains supplementary material, which is available to authorized users.

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## Introduction

*Colletotrichum* is among the most economically important genera of plant pathogenic fungi worldwide (Sutton 1992; Cai et al. 2009; Phoulivong 2011). Many species of *Colletotrichum* cause diseases of a wide range of important crops commonly known as anthracnose (Sutton 1992; Hyde et al. 2009a). In addition, many *Colletotrichum* species are latent plant pathogens, species essentially being endophytes, epiphytes or saprobes, switching to a pathogenic lifestyle when host plants are stressed or in postharvest storage (Hyde et al. 2009b).

The history of the naming of *Colletotrichum* species has recently been reviewed in several important papers (Cannon et al. 2008; Hyde et al. 2009a; Weir et al. 2012) and recent protocols for the identification of new species were outlined in Cai et al. (2009). Following adoption of these polyphasic protocols for studying the genus *Colletotrichum*, especially the use of multi-gene phylogenetic analysis, the classification and species concepts in *Colletotrichum* changed significantly (Cai et al. 2009; Cannon et al. 2012; Damm et al. 2012a, b; Weir et al. 2012). A systematic study of nearly all acknowledged species, revealed at least nine clades in this genus (Cannon et al. 2012); many species previously known as a single species proved to be polyphyletic taxa (Cannon et al. 2012). *Colletotrichum gloeosporioides* (Cannon et al. 2008; Phoulivong et al. 2010b; Weir et al. 2012), *C.*

*acutatum* (Marcelino et al. 2008; Shivas and Yu 2009; Damm et al. 2012a), *C. boninense* (Moriwaki et al. 2003; Yang et al. 2009; Damm et al. 2012b) and *C. truncatum* (Damm et al. 2009; Cannon et al. 2012) are important species complexes and well resolved among all the nine clades. Further studies in these species complexes; such as the newly published species, *C. murrayae* (Peng et al. 2012) and *C. viniferum* (Peng et al. 2013) in the *C. gloeosporioides* species complex improve our knowledge concerning *Colletotrichum* systematics.

The citrus industry is one of the most important fruit industries worldwide and therefore the study and knowledge of pathogens of this crop is very important (Wang et al. 2012). Three important diseases of *Citrus* caused by *Colletotrichum* species worldwide are anthracnose, postbloom fruit drop and key lime anthracnose (Timmer et al. 2000; Lima et al. 2011; McGovern et al. 2012). Anthracnose was previously thought to be caused by *C. gloeosporioides* (Sutton 1980) only, but recent work has shown that at least thirteen species of *Colletotrichum* are associated with *Citrus* in China (Peng et al. 2012) and other countries (Damm et al. 2012a, b; Weir et al. 2012). Postbloom fruit drop was thought to be caused by *C. acutatum*, but *C. gloeosporioides* was also found to be the causal agent of postbloom fruit drop in Brazil (Lima et al. 2011) and Bermuda (McGovern et al. 2012). Key lime anthracnose was reported to be caused by "*C. acutatum*" but the taxon was different from *C. acutatum* species causing postbloom fruit drop (Peres et al. 2008; MacKenzie et al. 2009) and now recognized as *C. limetticola* (R.E. Clausen) Damm, P.F. Cannon and Crous (Damm et al. 2012a). With the recent changes in understanding of species concepts in *Colletotrichum*, new surveys are required to identify the species causing *Citrus* diseases (Ko Ko et al. 2011b).

*Colletotrichum* diseases of *Citrus* in China had not been well researched, and thus *C. gloeosporioides* is generally recognized as one of the most important pathogens in all cultivated *Rutaceae* all over China (Peng et al. 2012). *Colletotrichum gloeosporioides* is reported to cause young shoot and leaf blight, leaf spot, stem-end wither followed by premature fruit drop and postharvest fruits anthracnose (Chinese Research Institute of Pomology and Citrus 1994). However, previous identifications were based on techniques available at the time with identification being based on examination of symptoms, and morphology of conidia produced on the infected tissues (Sutton 1992). Peng et al. (2012) however, showed that seven *Colletotrichum* species caused anthracnose of citrus leaves, while *C. acutatum*, which is the causal agent of postbloom fruit drop (Timmer et al. 1994) of many cultivated citrus species has not been reported in China.

The objective of the present study was to characterize *Colletotrichum* species associated with cultivated *Citrus* and *Fortunella* species in the major citrus growing areas in China. Accurate data on *Colletotrichum* species causing disease of citrus in China will allow quarantine officials, plant breeders and plant health practitioners to make informed decisions concerning import and export, epidemiology, research, and control and management of citrus diseases.

## Material and methods

### Isolation and culture

Material of both asymptomatic and diseased citrus organs were collected from the major Citrus growing areas in China, including Zhejiang, Jiangxi, Guangdong, Guangxi, Yunnan, Fujian and Shaanxi provinces. Isolation of *Colletotrichum* species from the diseased tissues followed the methods outlined by Cai et al. (2009). Symptomless and diseased tissues without sporulation were cut into small pieces and surface sterilized by dipping in 1 % sodium hypochlorite for 1 min, 70 % ethanol for 1 min and washed three times in sterile water and dried on sterilized filter paper. All pieces were placed on PDA plates and incubated until mycelium grew out. If conidial mass formed, the conidia were harvested and suspended in sterile water. Isolations were made directly from single conidium in the case of pathogens or saprobes were produced on the samples as detailed in Chomnunti et al. (2011). All isolates were grown at 25 °C on PDA for further study. Selected strains used for further study are listed in Table 1.

### Morphological study

Isolates were transferred from the actively growing edge of 4 day old colony by cutting small squares of agar with mycelium, plating on fresh PDA plates and incubating at 25 °C in 12/12 h fluorescent light and the dark. The growth rate was recorded by measuring the diameter of the colonies until day 5, and the mean growth rate was calculated per day. The colonies characters were also recorded. All experiments were preformed in triplicate. After 7 days, the shape, colour and size of 30 conidia and conidiophores was recorded (Eclipse 80i, Nikon, Japan). Appressoria was induced using a slide culture technique (Johnston and Jones 1997; Cai et al. 2009) and after 7 days, the shape, colour and size of 30 appressoria were recorded (Eclipse 80i, Nikon, Japan).

### DNA extraction, PCR amplification and sequencing

Isolates were grown for 4 days and surface mycelia were removed using a sterile scarpel blade. The collected mycelia were placed in centrifuge tubes and the genomic DNA was extracted using a Biospin Fungus Genomic DNA Extraction

**Table 1** *Colletotrichum* species isolated from *Citrus* species in China

| Species | Isolate NO. | Host | Symptoms | Origin | Year | Collector |
|---|---|---|---|---|---|---|
| *Colletotrichum gloeosporioides* | ZJUC1 | *Citrus sinensis* | asymptomatic leaf | Ganzhou, Jiangxi | 2011 | Feng Huang |
| | ZJUC2 | *C. sinensis* | asymptomatic leaf | Ganzhou, Jiangxi | 2011 | Feng Huang |
| | ZJUC3 | *C. unchiu* | asymptomatic branch | Ganzhou, Jiangxi | 2011 | Feng Huang |
| | ZJUC4 | *C. grandis* | asymptomatic branch | Ganzhou, Jiangxi | 2011 | Feng Huang |
| | ZJUC5 | *C. unchiu* | asymptomatic leaf | Linhai, Zhejiang | 2011 | Feng Huang |
| | ZJUC6 | *C. reticulata* cv. nanfengmiju | asymptomatic branch | Nanfeng, Jiangxi | 2011 | Feng Huang |
| | ZJUC7 | *C. reticulata* cv. nanfengmiju | asymptomatic branch | Nanfeng, Jiangxi | 2011 | Feng Huang |
| | ZJUC8 | *C. reticulata* cv. nanfengmiju | asymptomatic branch | Nanfeng, Jiangxi | 2011 | Feng Huang |
| | ZJUC9 | *C. reticulata* | asymptomatic branch | Quzhou, Zhejiang | 2011 | Feng Huang |
| | ZJUC10 | *C. reticulata* | asymptomatic branch | Quzhou, Zhejiang | 2011 | Feng Huang |
| | ZJUC11 | *C. reticulata* | asymptomatic branch | Quzhou, Zhejiang | 2011 | Feng Huang |
| | ZJUC12 | *C. unchiu* | Saprophytes on leaf | Chenggu, Shaanxi | 2012 | Feng Huang |
| | ZJUC13 | *C. unchiu* | Saprophytes on leaf | Chenggu, Shaanxi | 2012 | Feng Huang |
| | ZJUC14 | *C. unchiu* | asymptomatic branch | Chenggu, Shaanxi | 2011 | Feng Huang |
| | ZJUC15 | *C. unchiu* | asymptomatic branch | Chenggu, Shaanxi | 2011 | Feng Huang |
| | ZJUC17 | *C. reticulata* | Non-symptom leaf | Ruili, Yunnan | 2011 | Feng Huang |
| | ZJUC18 | *C. reticulata* | asymptomatic branch | Ruili, Yunnan | 2011 | Feng Huang |
| | ZJUC19 | *C. grandis* | asymptomatic leaf | Zhangzhou, Fujian | 2011 | Feng Huang |
| *C. fructicola* | ZJUC20 | *C. reticulata* cv. nanfengmiju | asymptomatic leaf | Nanfeng, Jiangxi | 2011 | Feng Huang |
| | ZJUC21=CBS134225 | *C. reticulata* cv. nanfengmiju | asymptomatic leaf | Nanfeng, Jiangxi | 2011 | Feng Huang |
| | ZJUC22 | *C. reticulata* cv. nanfengmiju | asymptomatic leaf | Nanfeng, Jiangxi | 2011 | Feng Huang |
| | ZJUC23 | *Fortunella margarita* | asymptomatic branch | Guilin, Guangxi | 2012 | Feng Huang |
| | ZJUC24 | *F. margarita* | asymptomatic branch | Guilin, Guangxi | 2012 | Feng Huang |
| | ZJUC25 | *F. margarita* | asymptomatic branch | Guilin, Guangxi | 2012 | Feng Huang |
| | ZJUC26 | *F. margarita* | asymptomatic branch | Guilin, Guangxi | 2012 | Feng Huang |
| *C. karstii* | ZJUC27=CBS134226 | *C. limon* | asymptomatic branch | Ruili, Yunnan | 2011 | Feng Huang |
| | ZJUC28 | *C. limon* | Anthracnose of leaf | Ruili, Yunnan | 2011 | Feng Huang |
| | ZJUC29 | *C. limon* | Anthracnose of leaf | Ruili, Yunnan | 2011 | Guoqing Chen |
| | ZJUC30 | *C. limon* | Anthracnose of leaf | Ruili, Yunnan | 2011 | Guoqing Chen |
| | ZJUC31 | *C. grandis* | asymptomatic leaf | Zhangzhou, Fujian | 2011 | Guoqing Chen |
| | ZJUC32=CBS134227 | *C. grandis* | asymptomatic shoot | Zhangzhou, Fujian | 2011 | Feng Huang |

**Table 1** (continued)

| Species | Isolate NO. | Host | Symptoms | Origin | Year | Collector |
|---|---|---|---|---|---|---|
| | ZJUC33 | *C. grandis* | asymptomatic branch | Zhangzhou, Fujian | 2011 | Feng Huang |
| *C. citricola* | ZJUC34=CBS134228=CGMCC3.15227 | *C. unchiu* | Saprophytes on leaf | Chenggu, Shaanxi | 2012 | Feng Huang |
| | ZJUC35=CBS134229 | *C. unchiu* | Saprophytes on leaf | Chenggu, Shaanxi | 2012 | Feng Huang |
| | ZJUC36=CBS134230 | *C. unchiu* | Saprophytes on leaf | Chenggu, Shaanxi | 2012 | Feng Huang |
| *C. truncatum* | ZJUC37=CBS134231 | *C. flamea* | Anthracnose of shoot | Guilin, Guangxi | 2008 | Guoqing Chen |
| | ZJUC38=CBS134232 | *C. limon* | Anthracnose of leaf | Ruili, Yunnan | 2008 | Guoqing Chen |
| | ZJUC39 | *C. limon* | Anthracnose of leaf | Ruili, Yunnan | 2008 | Guoqing Chen |
| | ZJUC40 | *C. limon* | Anthracnose of leaf | Ruili, Yunnan | 2008 | Guoqing Chen |
| *C. citri* | ZJUC41=CBS134233=CGMCC3.15228 | *C. aurantifolia* | Anthracnose of shoot | Ruili, Yunnan | 2008 | Guoqing Chen |
| | ZJUC42=CBS134234 | *C. aurantifolia* | Anthracnose of shoot | Ruili, Yunnan | 2008 | Guoqing Chen |
| | ZJUC43=CBS134235 | *C.. aurantifolia* | Anthracnose of shoot | Ruili, Yunnan | 2008 | Guoqing Chen |

Kit (Bio-Flux, Bioer Technology Co., China) (Wang et al. 2012). Final DNA was suspended in 1× TE buffer and stored at −20 °C. PCR amplification was carried out in S1000TM Thermal Cycler (Bio-Rad Laboratories, Germany). The primers used in this study and their references are listed in Table 2. The protocols for amplification followed previous studies (Su et al. 2011). The amplicons were verified by staining with gelview (BioTeke Corporation, Beijing) and separated on 1 % agarose electrophoresis gel. Predicted fragments were collected and purified. The products were recombined into pMD18-T vector (TaKaRa Biotech. Co., Dalian, China), and transferred to *E. coli* (TG1) for proliferation. A single clone colony was harvested and the amplified product was sequenced. Sequences were edited by Lasergene 7 (DNAstar, USA).

## Phylogenetic analysis

Multi-locus sequences were prepared for phylogenetic analysis, the accession numbers of newly submitted and cited sequences from GenBank are listed in Online Resource 1. Sequences were aligned using Clustal X (Larkin et al. 2007), the aligned file was analyzed using the criterion of Maximum Parsimony (MP) in PAUP* 4b10 (Swofford

**Table 2** Primers used in this study

| Gene | Product | Primer | Sequence(5′–3′) | Reference |
|---|---|---|---|---|
| ACT | Actin | ACT-512F<br>ACT-783R | ATGTGCAAGGCCGGTTTCGC<br>TACGAGTCCTTCTGGCCCAT | Carbone and Kohn 1999 |
| TUB2 | β-tublin 2 | Bt2a<br>Bt2b | GGTAACCAAATCGGTGCTGCTTTC<br>ACCCTCAGTGTAGTGACCCTTGGC | Glass and Donaldson 1995 |
| CAL | Calmodulin | CL1<br>CL2 | GARTWCAAGGAGGCCTTCTC<br>TTTTTGCATCATGAGTTGGAC | O'Donnell et al. 2000 |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | GDF<br>GDR | GCCGTCAACGACCCCTTCATTGA<br>GGGTGGAGTCGTACTTGAGCATGT | Templeton et al. 1992 |
| GS | Glutamine synthetase | GSF1<br>GSR1 | ATGGCCGAGTACATCTGG<br>GAACCGTCGAAGTTCCAC | Stephenson et al. 1997 |
| ITS | Internal transcribed spacer | ITS4<br>ITS5 | TCCTCCGCTTATTGATATGC<br>GGAAGTAAAAGTCGTAACAAGG | White et al. 1990 |

2003). Trees were produced using a heuristic search option with random sequences addition at 1000. Maxtree number was unlimited and all trees were saved. Descriptive tree statistics were recorded, including tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI). The examination and evaluation of trees followed the methods reported in Yang et al. (2009) and Su et al. (2011). Trees are shown in Figtree (Rambaut and Drummond 2010).

### Pathogenicity tests

One representative strain from each species except *C. karstii* and *C. citricola* was selected for pathogenicity testing. Citrus fruits (Ponkan, *Citrus reticulata* Blanco) were transported to the laboratory directly from an orchard in Quzhou, Zhejiang Province, China. Non-wounded fruits were selected and surface-sterilized by immersing the fruits in 1 % sodium hypochlorite for 5 min, then washed completely in tap water and then dried in a fume hood. Conidial suspensions ($10^4$ conidia per ml) were prepared by growing the strains on PDA for 7 days and diluting the conidial masses with sterile water. A drop of 2.5-μl conidial suspension was added to a wound created by puncturing of the cortex with five sterile needles to about 0.5 mm depth. The fruits inoculated with sterile water were prepared as a negative control. The inoculated fruits were laid on the plastic tray, and the trays were covered with the plastic wrap to maintain the moisture, and incubated at 25 °C with 12/12 h fluorescent light and darkness. On each fruit, 1 to 4 sites (Fig. 5n) were inoculated, and the site was regarded as infected when the necrotic spot was significantly larger than that of negative control. At least ten fruits were inoculated for each strain, the trial was carried out in triplicate. The incidence of infection was calculated by the formula [Incidence (%) = (infected sites or fruits/inoculated sites or fruits)× 100 %] at 12-day post inoculation. The significant difference was analyzed with a LSD test by SAS 9.1 TS level 1 M3 (Inc. 2002–2004) software.

## Results

### Collection of *Colletotrichum* species

In total, 312 *Colletotrichum* strains were isolated from citrus tissues from the main citrus growing regions in China; 212 strains (68 %) were isolated from diseased tissues, 70 strains (22.4 %) from asymptomatic tissues and 30 strains (9.6 %) were saprobes. Based on morphology on PDA, most strains produced conidia similar to *C. gloeosporioides* (Fig. 5a-d). Seven strains produced the "Glomerella" sexual stage (Fig. 5e-n). Eleven strains produced conidia with a hilum or scar at the base (Fig. 6g, h); this is typical of the *C. boninense* species complex (Damm et al. 2012b). Four strains produced curved conidia (Fig. 7c), three strains produced fusiform conidia (Fig. 8b) which is typical for many species in the *C. acutatum* species complex (Damm et al. 2012a). Based on these morphological features, representative strains were selected for phylogenetic analysis and further taxonomic study.

### Phylogenetic analysis

We selected 38 strains for molecular analysis which included 24 strains from the *C. gloeosporioides* complex, nine strains from the *C. boninense* complex, two strains with curved conidia and three strains from *C. acutatum* species complex. Figure 1 is the phylogram calculated to identify the strains in the *C. gloeosporioides* species complex. The Kahawae and Musae clades could be distinguished, similar to the finding of Weir et al. (2012). Eighteen strains could be confidently identified as *C. gloeosporioides* as they clustered together with the ex-epitype strain CBS 953.97 with 100% bootstrap support. Six other strains clustered with *C. fructicola* strains (including the ex-type culture: ICMP 18581) with 99% bootstrap support in the Musae clade. Figure 2 was calculated to identify the strains placed in the *C. boninense* species complex. Six strains clustered with two representative strains (including the ex-type culture: CORCG6) of *C. karstii* with robust support (93%), while three strains (ZJUC34, ZJUC35, ZJUC36) appear in a distinct clade and could not be assigned to any currently known species. Two strains producing curved conidia clustered with *C. truncatum* (including the ex-type type: CBS 151.35) strains with 100% bootstrap support (Fig. 3). Figure 4 was calculated to identify three strains producing elliptical or fusiform conidia. These strains clustered in a distinct clade (100% bootstrap support) and were closest to *C. nymphaeae* strains (87% bootstrap support).

## Taxonomy

***Colletotrichum gloeosporioides*** (Penz.) Penz. & Sacc. Fig. 5a-d

Of the 312 strains isolated from *Citrus sinensis*, *C. unchiu*, *C. grandis*, *C. reticulata* and other *Citrus* species, 287 strains (92 %) were identified as *C. gloeosporioides sensu stricto*. These strains were isolated as pathogens (202 strains), endophytes (58 strains) and saprobes (27 strains). The conidia of the *C. gloeosporioides* species varied, strain ZJUC15 was generally shorter, being 11.3–14.7×5.3–6.4 μm ($\bar{x} = 12.6 \times 5.8\,\mu m$, Fig. 5a); ten strains produced conidia with two guttules

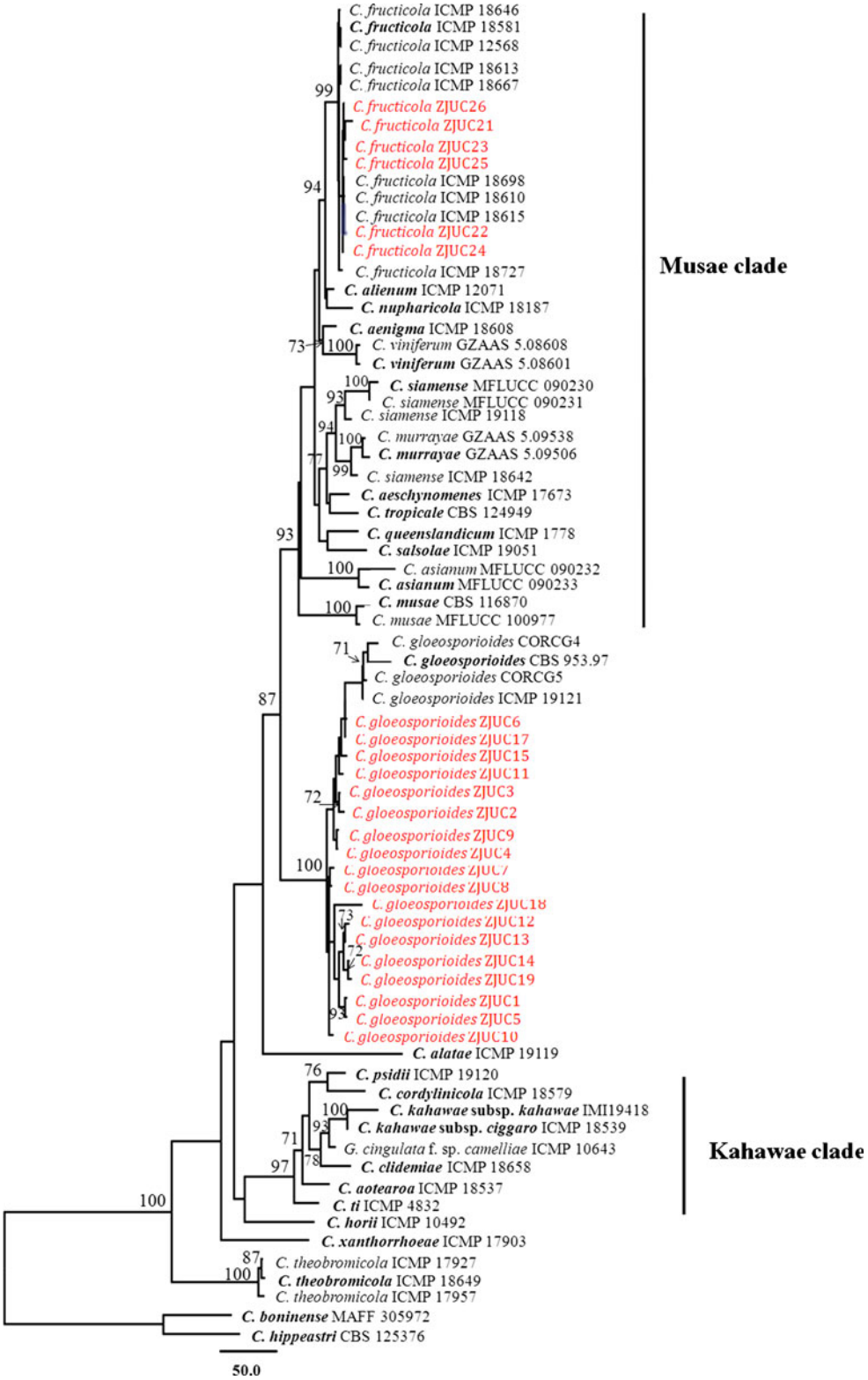


**Fig. 1** One of 1000 phylograms of the *Colletotrichum gloeosporioides* species complex based on maximum parsimony analysis with combined actin (ACT), β-tubulin (TUB2), calmodulin (CAL), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glutamine synthetase (GS) and internal transcribed spacer (ITS) sequences. Tree Length=1942, CI=0.730, RI=0.865, RC=0.631, HI=0.270. *C. boninense* and *C. hippeastri* are selected as the outgroup, ex-type and ex-epitype cultures are emphasized in bold

(Fig. 5b), ZJUC17 and ZJUC18 were somewhat fusiform (Fig. 5c), and ZJUC5 and ZJUC19 unusually large (Fig. 5d).

***Colletotrichum fructicola*** Prihastuti, L. Cai & K. D. Hyde Fig. 5e-n

*Colonies* growing about 7 mm in diameter each day at 25°C, olive to grey with white edge, stroma or sclerotia readily formed on PDA in 20 days. *Ascomata* 50–116 μm abundant, brown, globose, with a neck, and mostly sterile. *Asci* 34–70.3×7.6–11 μm ($\bar{x} = 51 \times 8.8\,\mu m$), unitunicate

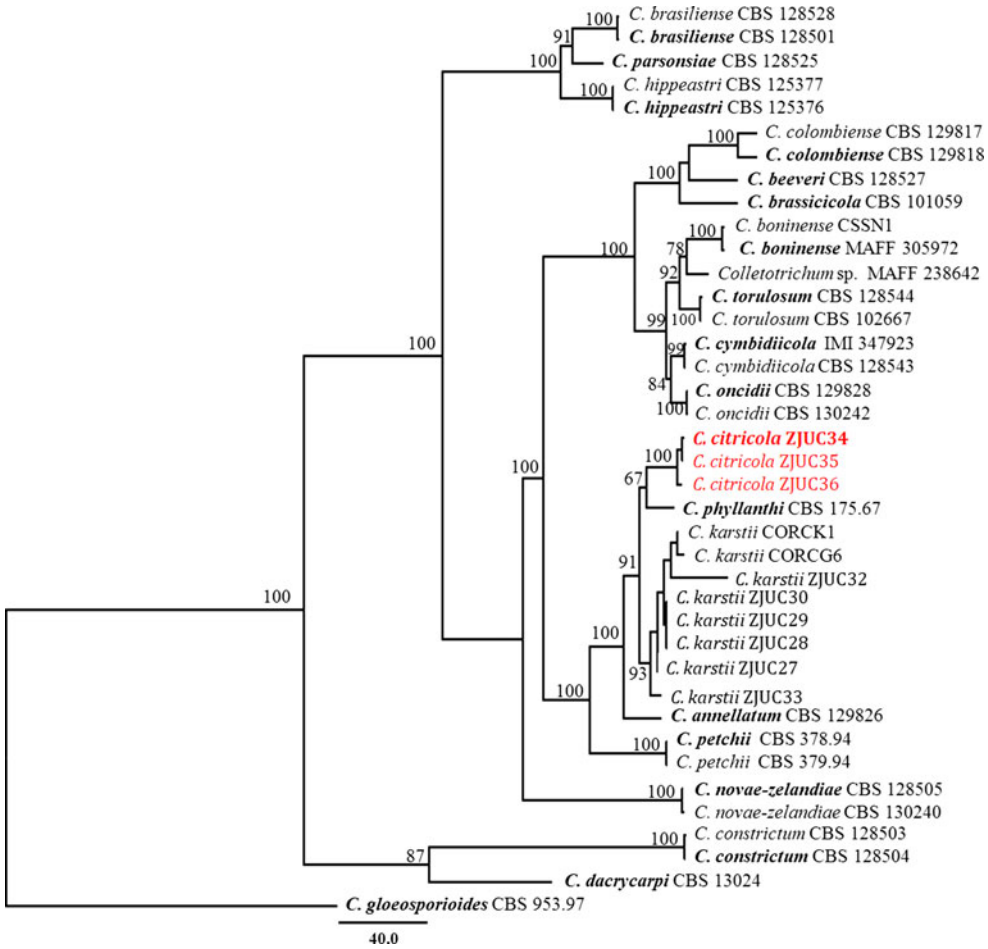


**Fig. 2** One of 11 phylograms of the *Colletotrichum boninense* species complex based on maximum parsimony analysis with combined actin (ACT), β-tubulin (TUB2), calmodulin (CAL), chitin synthase 1 (CHS-1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and internal transcribed spacer (ITS) sequences. Tree Length= 1218, CI=0.750, RI=0.877, RC=0.658, HI=0.250. *C. gloeosporioides* strain CBS 953.97 is selected as the outgroup, ex-type or ex-epitype cultures are emphasized in bold

and clavate to cymbiform. *Ascospores* 11–17.4×2.7–4.7 μm ($\overline{x}$ = 14.4 × 3.6 μm), one-celled, hyaline, guttulate, fusiform to slightly curved and with rounded ends. *Conidiomata* rarely observed. *Conidia* 12.9–18.9×4.5–6.2 μm ($\overline{x}$ = 15.5 ×5.4 μm), in brick-red masses, hyaline, cylindrical, with rounded ends. *Appressoria* 8.2–16.4×4–8.8 μm ($\overline{x}$ = 12 ×5.6 μm), formed from mycelia, brown to dark brown, oval to clavate, sometimes with one end pointed, ovoid or irregular.

*Habitat:* endophytes from *Citrus reticulata* cv. nanfengmiju and *Fortunella margarita* (Lour. ) Swingle.

*Notes:* Compared to the original description of *C. fructicola* (Prihastuti et al. 2009), where conidia were 3–4.3 μm wide, strain ZJUC22 was 4.5–6.2 μm wide, strain ZJUC26 was 4.4–5.6 μm wide and strain ZJUC24 was 4.2–5.8 μm wide; thus all strains in this study were wider.

***Colletotrichum karstii*** Y.L. Yang, Z.Y. Liu, K.D. Hyde & L. Cai

Six strains of this species were isolated, three from anthracnose spots on leaves, the other three from asymptomatic tissues of *Citrus*.

***Colletotrichum citricola*** F. Huang, L. Cai, K.D. Hyde & H.Y. Li, **sp. nov.** Fig. 6
Mycobank: MB 803017

*Etymology:* in reference to its occurrence on Citrus = *citricola*

*Holotype:* Chenggu, Shaanxi province, China, Saprobes on leaf of *Citrus unshiu*, May 2012, F. Huang (ZJUC34H , holotype), culture ex-type, ZJUC34=CBS134228=CGMCC3.15227; Chenggu, Shaanxi province, China, Saprobes on leaf of *Citrus unshiu*, May 2012, F. Huang, ZJUD35=CBS134229, ZJUC36=CBS134230.

When grown on PDA, *colonies* orange, mycelia loose. Stroma observed in about 1 week. *Ascomata* orange to brown, globose to near globose. *Asci* 61×13 μm, unitunicate, clavate. *Ascospores* 12.8–18.4×5.3–6.7 μm, ($\overline{x}$ = 15.8 × 6.1 μm), one-celled, hyaline, slightly curved to cylindrical, rounded at the ends. *Conidiomata* barely observed, conidia formed in orange masses, conidiophores hyaline, branched. *Conidiogenous cells* 14.9–31.8×2.8–4.3 μm ($\overline{x}$ = 20.6 × 3.6 μm), hyaline, tapered at the apex. *Conidia* 13.7–16.1×5.9–6.9 μm, ($\overline{x}$ = 15.1 ×6.4 μm), cylindrical, hyaline, rounded at two ends, often with

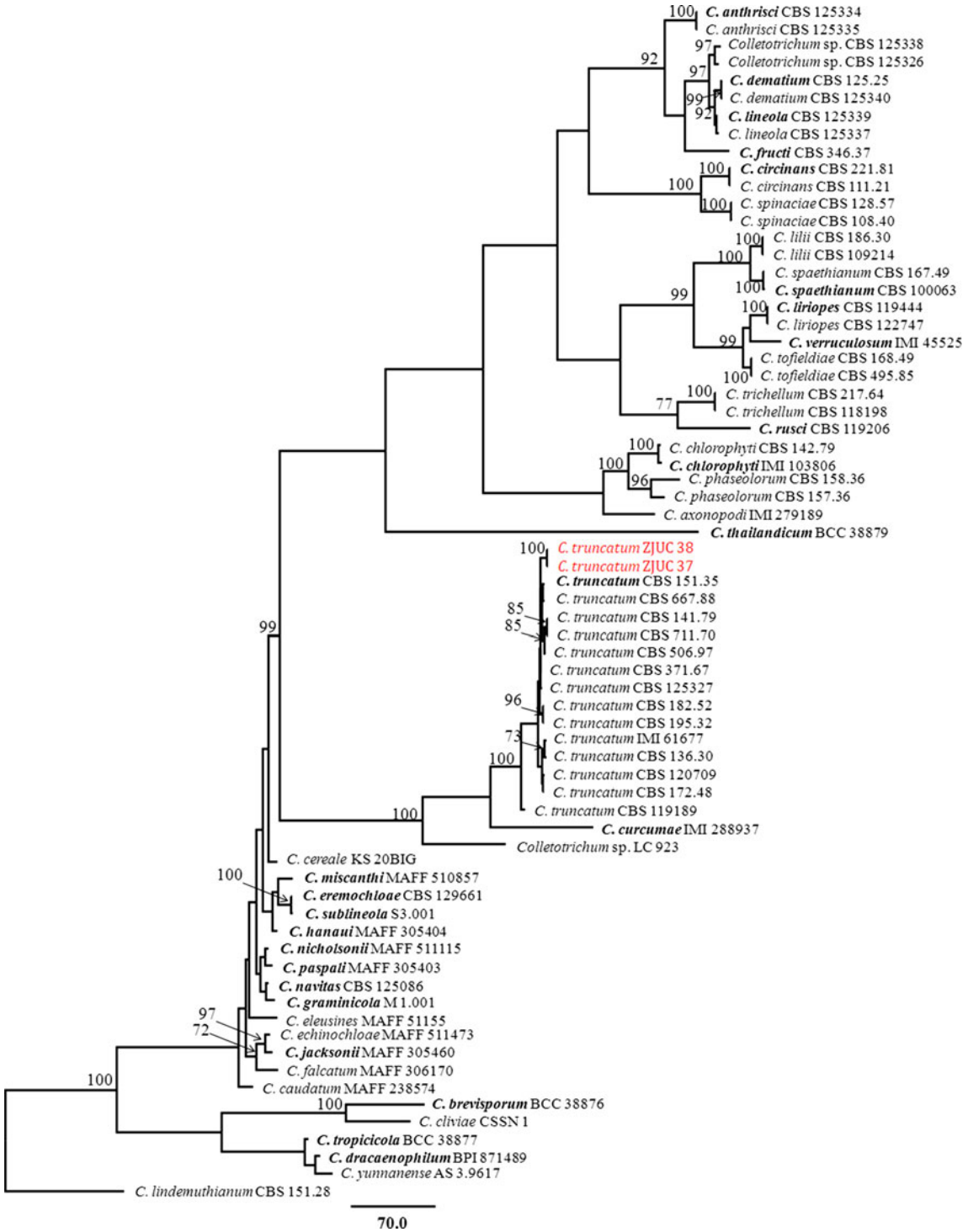


**Fig. 3** One of 1000 phylograms of the *Colletotrichum truncatum* species complex based on maximum parsimony analysis with combined actin (ACT), β-tubulin (TUB2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and internal transcribed spacer (ITS) sequences. Tree Length=2817, CI=0.580, RI=0.863, RC=0.501, HI=0.420. *C. lindemuthianum* was selected as the taxon, ex-type and ex-epitype cultures are emphasized in bold

one end wider, sometimes with a hilum-like base. *Appressoria* 5.8–10.9 μm diam. ($\bar{x}$ = 8.2 μm), brown, roundish.

*Habitat:* Saprobes on *Citrus unchiu* Hort. ex Tanaka.

*Known distribution:* Chenggu, Shaanxi Province, China.

*Notes:* This species belongs to the *C. boninense* species complex (Fig. 2) and clustered with *C. phyllanthi* with relative low support (67%). All three strains form a distinct clade with 100% bootstrap support indicating they represent a distinct

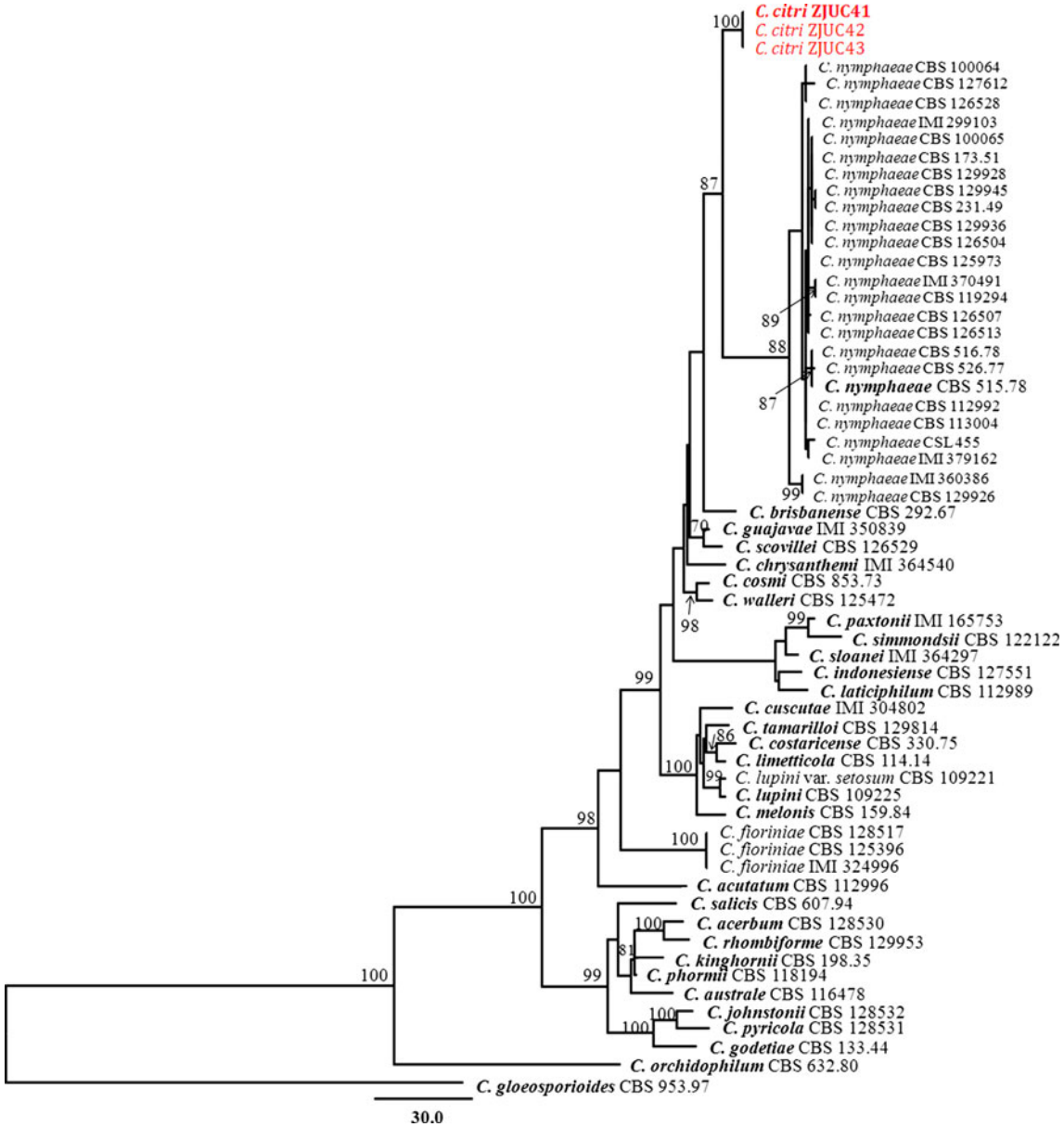


**Fig. 4** One of 12 phylograms of the *Colletotrichum acutatum* species complex from maximum parsimony analysis based on actin (ACT), β-tubulin (TUB2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and internal transcribed spacer (ITS) sequences. Tree Length=853, CI=0.769, RI=0.844, RC=0.649, HI=0.231. *C. gloeosporioides* strain CBS 953.97 was selected as the outgroup, ex-type or ex-epitype culture are emphasized in bold

species. Therefore, *C. citricola* is introduced to accommodate this new species. *Colletotrichum phyllanthi* differs from *C. citricola* by its narrower conidia at 3–5 μm (Pai 1970) versus 5.9–6.9 μm in *C. citricola*.

***Colletotrichum truncatum*** (Schwein.) Andrus & W.D. Moore Fig. 7

*Colonies* growing 10–13 mm diameter each day at 25 °C, after 8 days, flat, grey at the center from above, with obvious olive to green margin; in reverse grey to black with radial mycelia growth. *Stroma* formed and masses of saffron conidia observed. *Conidia* 23.1–29.6×2.6–3.9 μm ($\overline{x}$ = 26.3 × 3.4 μm), hyaline, aseptate, smooth walled, slightly curved at the centre with hook-like ends. *Setae* on leaf spot, 171×4.3 μm, brown, tapered to the apex.

***Colletotrichum citri*** F. Huang, L. Cai, K.D. Hyde & H.Y. Li, **sp. nov.** Fig. 8
Mycobank: MB 803017

*Etymology:* from *Citrus* in reference to the occurrence on this host.

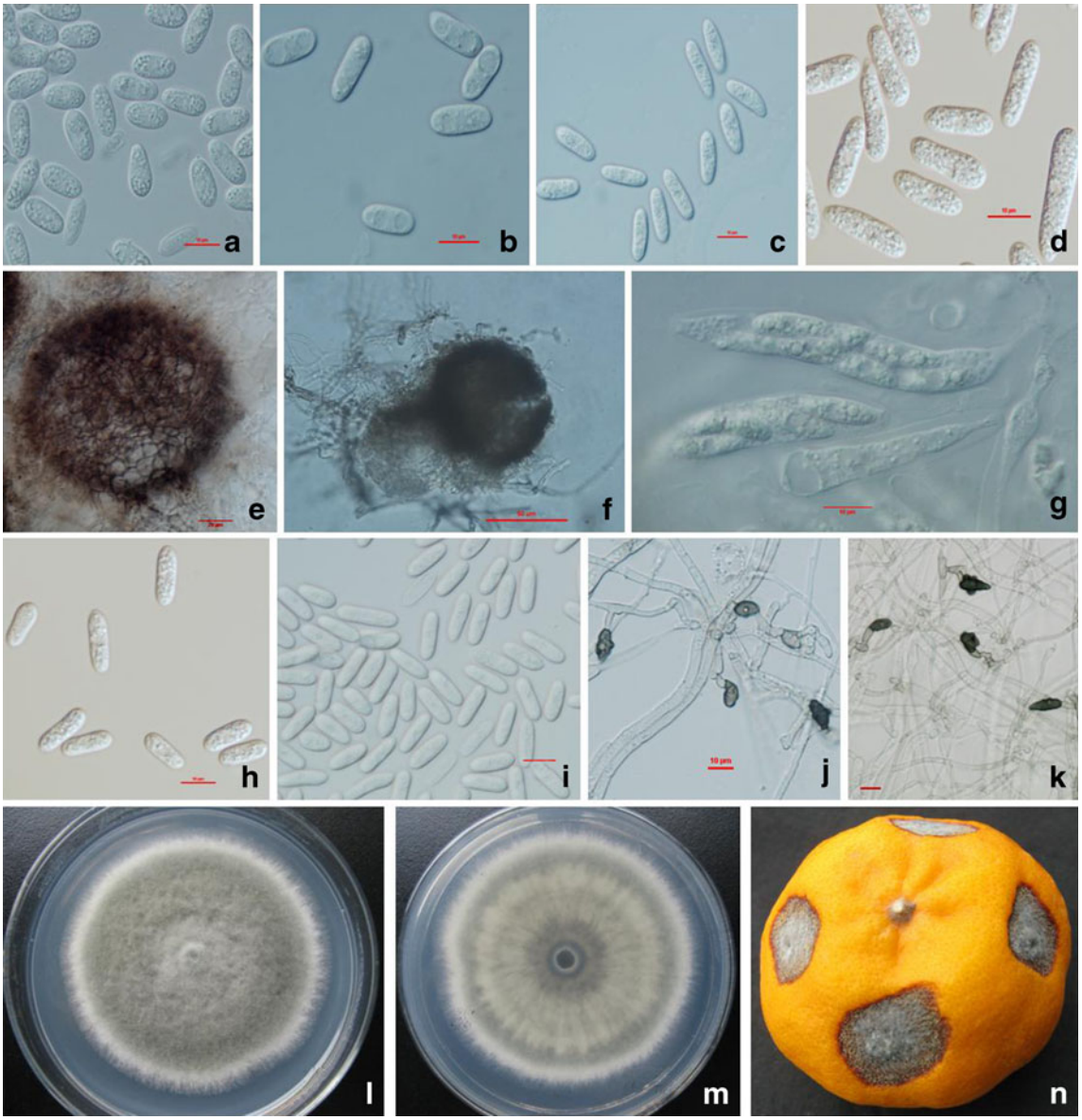


**Fig. 5** *Colletotrichum gloeosporioides*: **a**, Conidia of ZJUC15; **b**, Conidia of ZJUC10; **c**, Conidia of ZJUC18; **d**, Conidia of ZJUC5. *Colletotrichum fructicola* (ZJUC22): **e** and **f**, Ascocarps. **g**, Asci. **h**, Conidia. **i**, Conidia (from ZJUC24). **j** and **k**, Appressoria. **l**, Colonies on PDA agar medium above. **m**, Colonies on PDA agar medium below. **n**, Induced symptoms on fruit (*Citrus reticulata*). Scale: E= 20 μm, F=50 μm, others=10 μm

*Holotype:* Ruili, Yunnan province, China, on Anthracnose of a shoot of *Citrus aurantifolia*, August 2008, G. Q. Chen (ZJUC41H, holotype), culture ex-type, ZJUC41=CBS 134233=CGMCC3.15228; Ruili, Yunnan province, China, on Anthracnose shoot of *Citrus aurantifolia*, August 2008, G. Q. Chen, ZJUC42=CBS134234 and ZJUC43=CB S134235.

*Colonies* growing 6–8 mm in diameter each day at 25 °C with 12/12 alteration between fluorescent light and dark. After 8 days, the colony was grey and flat from above, and grey to black from below. When inoculated on PDA, stromata produced in the surface of culture, setae absent, light red masses of conidia formed. *Conidia* 9.5–13.7×3.3–4.4 μm ($\overline{x} = 12 \times 3.9$ μm), hyaline, smooth, acute to near rounded at two ends. *Appressoria* 8×4.5 μm diam, produced from mycelia, light brown to brown, roundish to ellipsoidal. Sexual state not observed.

*Habitat:* ZJUC41, ZJUC42 and ZJUC43 were isolated from the shoots of Key Lime (*Citrus aurantifolia*), causing black withered symptoms.

*Known distribution:* Ruili, Yunnan Province, China.

*Notes:* This species is similar to the three species clarified by Shivas and Yu (2009), when the author relied on the

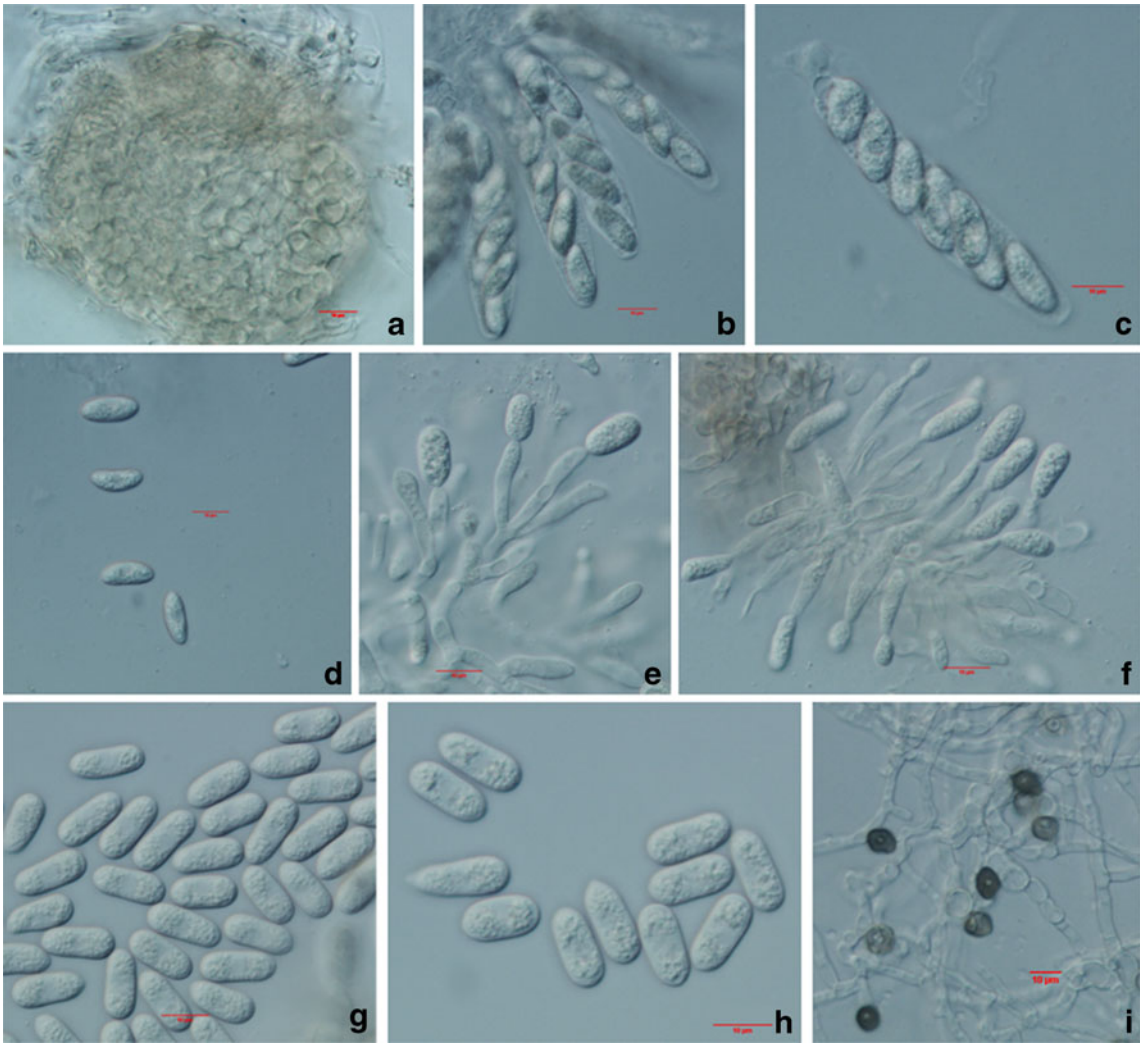


**Fig. 6** *Colletotrichum citricola* (ZJUC35). **a**. Ascocarp. **b** and **c**. Asci. **d**. Ascospores. **e** and **f**. Conidiophores. **g** and **h**. Conidia. **i**. Appressoria. Scale=10 μm

differences of interspecies on colonial characteristics and phylogenetic analysis. This species can be differed with *C. nymphaeae* (Johnson et al. 1997) and *C. limetticola* (Damm et al. 2012a) by conidia shape and size, while *C. citri* conidia was shorter and narrower on average.

## Pathogenicity testing

The result of pathogenicity testing is shown in Table 3. *Colletotrichum gloeosporioides* representative (strain ZJUC17) was virulent on most experimental fruits with a mean infection incidence of 93%. *Colletotrichum fructicola* (strain ZJUC22) (Fig. 5n) infected experimental fruits with a lower mean infection incidence (90%) but this was not significantly different to ZJUC17. *Colletotrichum truncatum* (strain ZJUC37) was also pathogenic to *Citrus* fruit with a mean infection incidence of 83% on experimental fruits (Fig. 7f). *Colletotrichum citri* strain ZJUC41 showed no virulence to *Citrus* fruits, but it could infect the petal by inoculation (data not shown). *Colletotrichum citri* is not common on *Citrus* as only three strains were obtained in this study.

**Table 3** Pathogenicity testing of *Colletotrichum* species from *Citrus*

| Species | Strain | Mean infection incidence (%) | t-grouping |
|---|---|---|---|
| *C. gloeosporioides* | ZJUC17 | 93% | A |
| *C. fructicola* | ZJUC22 | 90% | A |
| *C. truncatum* | ZJUC37 | 83% | A |
| *C. citri* | ZJUC41 | 0 | B |
| control | $H_2O$ | 0 | |
| LSD($P$=0.05) | | 0.116 | |

t-grouping significant difference at $P= 0.05$ level are indicated by different letters

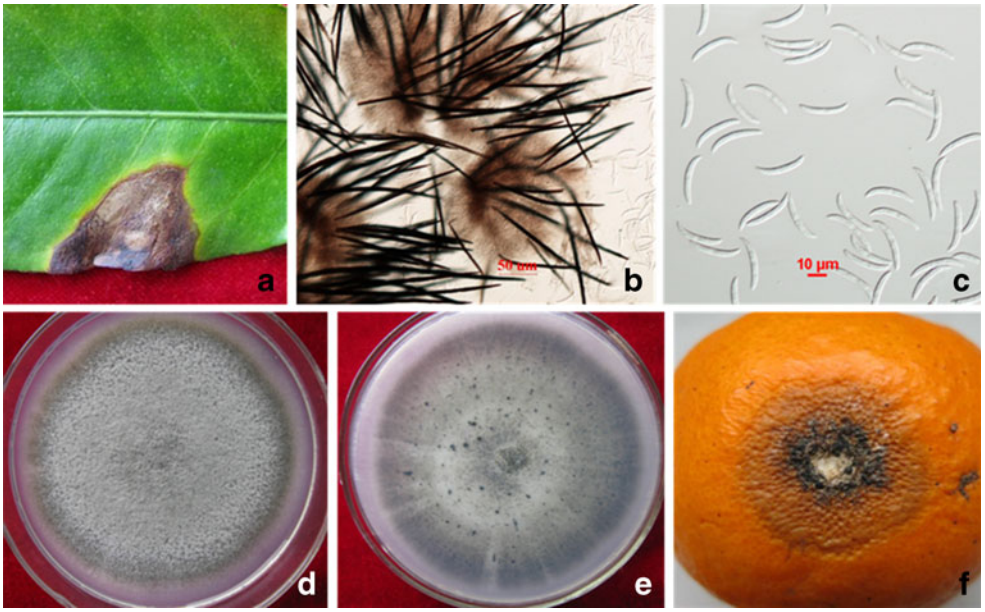


**Fig. 7** *Colletotrichum truncatum* (ZJUC37). **a**. Anthracnose symptom caused on leaf of Lemon (*Citrus limon*). **b**. Setae in clusters. **c**. Conidia. **d**. Colony form upside. **e**. Colony form downside. **f**. Inoculation induced symptom on citrus fruit (*Citrus reticulata*). Scale: B= 50 μm, C=10 μm

## Discussion

Previous studies on *Colletotrichum* species causing *Citrus* disease used morphological and single gene based identifications and thus taxa would have been identified to species complexes rather than individual species. A typical study to identify the diseases of *Citrus* caused by *Colletotrichum* species using multi-locus data was that of Peng et al. (2012) who isolated seven species of *Colletotrichum* from diseased *Citrus* leaves in Guizhou and Yunnan provinces in China.

In this study we surveyed a much wider area of China, and sampled more *Citrus* varieties and tissues and obtained more *Colletotrichum* strains. Based on multi-locus data we found that *Colletotrichum gloeosporioides* in the *C. gloeosporioides* species complex was a predominant species. It infects the main cultivated *Citrus* species in China, including *Citrus sinensis, C. unchiu*, *C grandis*, *C. reticulata* and *C. limon*. *Colletotrichum fructicola* also in the *C. gloeosporioides* species complex was only obtained from symptomless tissues. However, pathogenicity tests showed that both species can cause disease of *Citrus* fruits, indicating they could switch their lifestyle from endophyte to pathogen when the host is wounded or mature. Among the two species differentiated in the *C. boninense* species complex in this study, *C. karstii* was previously found to be an important pathogen on *Orchidaceae* hosts (Yang et al. 2011), and has also been isolated from *Citrus* plants in South Africa, New Zealand (Damm et al. 2012b) and China (Peng et al. 2012). Peng et al. (2012) showed that this

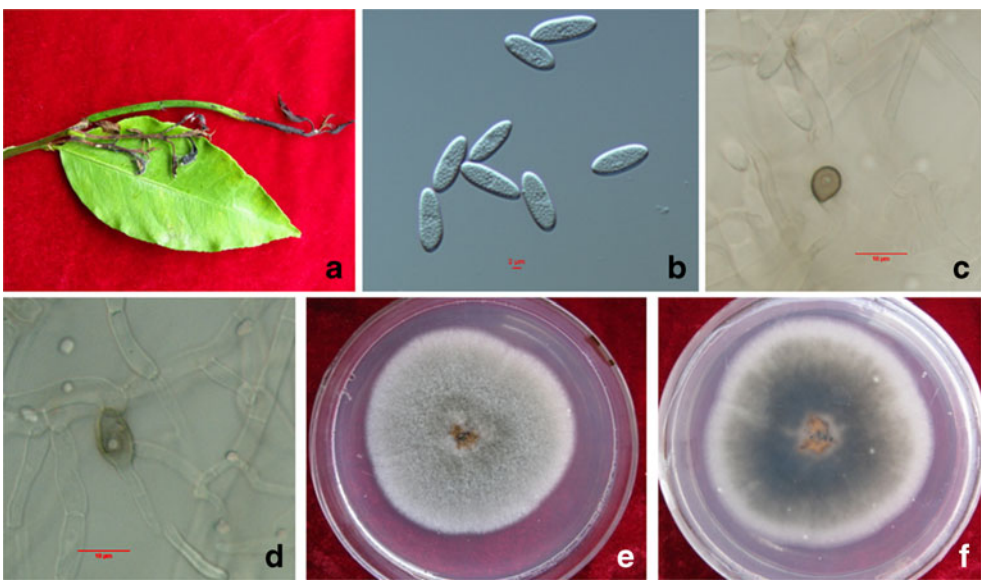


**Fig. 8** *Colletotrichum citri* (ZJUC41). **a**. Symptom caused on Lime shoot. **b**. Conidia. **c**, **d**. Appressoria. **e**. Colonies on PDA agar medium above. **f**. Colonies on PDA agar medium below. Scale: A=2 μm, C and D=10 μm

species cause *Citrus* leaf anthracnose. *Colletotrichum citricola* is a new species isolated from leaf anthracnose in the northwestern *Citrus* cultivation areas of China. *Colletotrichum truncatum* may also be a potential pathogen of *Citrus* species as shown in the pathogenicity tests. This species occurs in herbaceous plants (Damm et al. 2009), but this is the first report on *Citrus* spp.. *Colletotrichum citri,* a new species with fusiform conidia in the *C. acutatum* species complex (Damm et al. 2012a), was closest to *C. nymphaeae* (Van der Aa 1978).

*Colletotrichum* comprises species that can infect several host genera, such as *C. siamense* (Prihastuti et al. 2009; Yang et al. 2009; Wikee et al. 2011). On the other hand, a single host can harbor several species of *Colletotrichum* (Peng et al. 2012; Peng et al. 2013). Some species of *Colletotrichum*, however, appear to be specific to a single host species or genus (Prihastuti et al. 2010; Liu et al. 2011; Su et al. 2011)

*Colletotrichum gloeosporioides* was previously thought to be a common pathogen of numerous crops and most tropical fruits (Sutton 1992; Cannon et al. 2008). However, epitypification of this species by Cannon et al. (2008) has allowed for accurate identification of the taxon using analysis of multi-locus molecular data. Subsequent studies by Phoulivong et al. (2010a) have shown that *C. gloeosporioides* is not the common tropical pathogen as once believed (Phoulivong et al. 2010a). Since multi-locus analysis of *Colletotrichum* disease showed this species had been common on *Citrus* where it causes anthracnose, but rare on most other hosts (Prihastuti et al. 2009; Yang et al. 2009; Wikee et al. 2011; Weir et al. 2012; Peng et al. 2013).

In this study we have determined the species of *Colletotrichum* that cause disease of *Citrus* in the main growing areas of China. The most important causal agent is *C. gloeosporioides*. *C. acutatum* has not been found in China, probably because we did not survey in flowering season, and the collected strains were from the infected petals. In this study pathogenicity testing was only carried out on mature *Citrus* fruits by wound inoculation. The virulence and potential threat of these species to cause disease of leaves, shoots and petals needs to be clarified (Ko Ko et al. 2011a; Ko Ko et al. 2011b).

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