Diversity of Colletotrichum Species Causing Apple Bitter Rot and Glomerella Leaf Spot in China

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Abstract: Bitter rot and Glomerella leaf spot (GLS) of apples, caused by Colletotrichum species, are major diseases of apples around the world. A total of 98 isolates were obtained from apple fruits with bitter rot, and 53 isolates were obtained from leaves with leaf spot in the primary apple production regions in China. These isolates were characterized morphologically, and five gene regions (ITS, ACT, GAPDH, CHS-1 and TUB2) were sequenced for each isolate. A phylogenetic analysis, combined with a comparison of the morphological, cultural and pathogenic characters, sorted bitter rot isolates into six species: C. alienum, C. fructicola, C. gloeosporioides sensu stricto, C. nymphaeae, C. siamense and one new species, C. orientalis. Among these, C. siamense was the predominant pathogen associated with bitter rot. Isolates from leaf spot were identified as two species, C. aelegma and C. fructicola. This is the first report of C. orientalis as an apple bitter rot pathogen worldwide, and the results provide important insights into the diversity of Colletotrichum species in China.

Keywords: apple bitter rot; Colletotrichum; Glomerella leaf spot; Malus

1. Introduction

Apple bitter rot (ABR) is a common pre- and post-harvest disease in nearly all apple-growing areas worldwide. Because of its latent infection ability, crop losses can be severe from mid- to late-summer under prolonged warm and wet weather conditions [1]. The earliest record of a pathogen causing ABR is from 1856 when Gloeosporium fructigenum was described as the causal agent [2]. The fungus causing ABR was renamed several times until all species became synonymous to Glomerella cingulata (Stoneman) Spauld. & H. Schrenk (anamorph: Colletotrichum gloeosporioides (Penz.) Penz. & Sacc.) in 1903. In 1965, C. acutatum J. H. Simmonds was distinguished from C. gloeosporioides based on physiology and morphology [3]. ABR pathogens were mainly reported to be C. gloeosporioides, G. cingulata and C. acutatum [4]. Jones et al. found that C. acutatum and C. gloeosporioides were recovered from 81% and 19%, respectively, of 165 symptomatic fruits collected from orchards in western Michigan [5]. Shi et al. reported that C. acutatum was the most predominant species (70%) associated with ABR in orchards in Arkansas, North Carolina and Virginia [6]. Restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD) analyses indicated high intraspecific diversity [1,7–9], which, however, might reflect interspecific differences in the revised Colletotrichum taxonomic system [10]. In addition to apple fruit bitter rot, Colletotrichum species also incur foliar disease, namely Glomerella leaf spot (GLS) [11]. GLS was first reported in Brazil in the 1980s and was subsequently reported in the USA and East Asia [1,7]. The disease causes severe leaf fall off...
on susceptible cultivars, such as Gala and Golden Delicious. The new Colletotrichum taxonomic system was established with polyphasic approaches with an emphasis on multigene phylogeny, in which ‘C. gloeosporioides’ and ‘C. acutatum’ are both monophyletic species complexes, with over 20 and 30 independent species, respectively [10,12]. Thus far, a number of ABR pathogenic species, belonging either to the C. acutatum species complex (CASC) (C. abscissum, C. acutatum, C. fioriniae, C. godetiae, C. melonis, C. nymphaeae and C. paranaense) or the C. gloeosporioides species complex (CGSC) (C. chrysophilum, C. fragariae, C. fructicola, C. gloeosporioides s. str., C. noveboracense, C. siamense, C. alienum and C. theobromicola) have been reported worldwide [13–27]. Compared with ABR, relatively few GLS pathogens have been recognized thus far; these include C. fructicola and C. aenigma, belonging to the CGSC; C. karstii, belonging to the C. boninense species complex (CBSC); and C. limetticola, belonging to the CASC [11,20,28–32].

In China, apple bitter rot occurs in almost all producing areas, and the pathogens have been identified as C. gloeosporioides and C. acutatum [33–35]. Unfortunately, these species may all represent species complexes. GLS is an emerging disease that was first reported in 2012, and the pathogens have been identified as C. fructicola and C. aenigma [28], yet hidden pathogen diversity may exist due to insufficient investigation. Therefore, the main objective of this study is to investigate the Colletotrichum species diversity associated with ABR and GLS in China; gaining this knowledge will provide clues towards more effective control measures against these devastating diseases.

2. Materials and Methods

2.1. Isolates

Isolates were collected from diseased apple tissues exhibiting bitter rot and leaf spot symptoms in commercial apple orchards in four provinces, including Liaoning, Shandong, Henan and Shaanxi, of China from 2009 to 2013. Small pieces of symptomatic tissue were cut from lesions, immersed in 70% alcohol for 1 min, rinsed with sterile water and then dried on sterilized filter paper before placement into Petri dishes with Potato Dextrose Agar (PDA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cultures were incubated for 4 days at 25 °C in darkness. A mycelial disc was taken from the actively growing edge of a mono-conidial colony, and then transferred onto new PDA plates. Monosporic isolates were obtained from the new cultures. The colony diameter, color of the conidial masses and zonation of the colony were recorded. Appressoria were induced using a slide culture technique, in which a 1 cm\(^2\) segment of PDA containing the isolate was placed in sterile water in a sterile Petri dish, covered with a sterile coverslip and incubated under high humidity at 25 °C in darkness. After 2 days, the shapes and sizes of 50 appressoria on the coverslip were recorded.

2.2. DNA Extraction and PCR Amplification

The protocol from Barnes et al. was used to extract DNA from the mycelia by scraping the surface of the PDA after it had been cultured for 7 days at 25 °C [37]. The quantity and quality of the DNA were estimated by UV microscopic spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). The partial rDNA-ITS, actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chitin synthase (CHS-1) and β-tubulin-2 (TUB2) genes were amplified by PCR using primer pairs of ITS1-F [38] + ITS4 [39], ACT-512F + ACT-783R [40], GDF1 + GDR1 [41], CHS-79F + CHS-354R [40] and Bt2a + Bt2b [42], respectively. The PCR protocols were performed as described by Damm et al. [43]. The sequences of the isolates described in this study were deposited in GenBank; the accession numbers are listed in Table 1.
2.3. Sequence Alignment and Phylogenetic Analysis

Preliminary alignments of the multi-locus sequences were conducted using Clustal X [44] with a manual adjustment and BioEdit for visual improvement wherever necessary. The concatenation of the five-gene sequences was completed in PhyloSuite [45]. A maximum likelihood (ML) analysis was performed by RAxML version 8 [46] under the GTR model [47], and a non-parametric bootstrap analysis with 1000 repetitions [48] was used to determine the statistical support of the phylogeny. Bayesian inference (BI) phylogeny construction was performed with MrBayes version 3.2.1 [49], with the GTR + G + I nucleotide substitution model. The analysis included two separate runs for $1 \times 10^7$ generations; each run was sampled every 1000 generations, and the convergence of all the parameters was checked using internal diagnostics. To construct the 50% majority-rule consensus tree, the first 25% generations were discarded as burn-in. The phylogenetic tree (Figure 1) was visualized using FigTree v 1.4.4. A potential recombination event between *C. fioriniae* and *C. orientalis* was detected based on a pairwise homoplasy index (PHI) analysis of the Genealogical Concordance Phylogenetic Species Recognition concept in SplitsTree version 4.11.3 using the multi-locus alignment dataset [50,51].

![Figure 1. Phylogram of the Colletotrichum species resulting from maximum likelihood and Bayesian analyses based on the combined alignment dataset of ITS, ACT, GAPDH, CHS-1 and TUB-2 sequences. Bootstrap support values above 60% and Bayesian posterior probability values above 0.9 were given at the nodes. Isolates isolated in this study and type strains are shown in bold. * Ex-holotype, ex-neotype, ex-epitype strains.](image-url)
2.4. Pathogenicity Tests

Twelve representative isolates of *Colletotrichum* were chosen based on species identity and locations. Healthy apple fruits and leaves were selected, washed with tap water, blown dry in the hood and surface-sterilized with 70% ethanol prior to inoculation. Leaves and fruits were drop-inoculated with the conidia suspension (approximately 10^6/mL in concentration) or mycelial plugs. The fruits’ wounds were made by sterile insect needles with about 10 holes within a circular area of 5 mm in diameter. After inoculation, the fruits were incubated at 25 °C in plastic bags. The disease incidence of each fungal isolate was recorded 3 days after inoculation. For each isolate, at least five fruit/leaf inoculation replicates were performed in each experiment, and the inoculation experiment was repeated two times.

Table 1. Fungal isolates and sequences used in the phylogenetic analysis of this study.

| Species       | Type Strain | Host       | County          | GenBank No.          |
|---------------|-------------|------------|-----------------|----------------------|
| *C. acerbum*  | CBS 128530  | Malus domestica | New Zealand | JQ948459 JQ949780 JQ498790 JQ499120 JQ950110 |
| *C. acutatum* | CBS 112996  | Carica papaya | Australia      | JQ005776 JQ005839 JQ498677 JQ005979 JQ005860 |
| *C. aesthyrcophone* | ICMP 18680 | Persia americana | Israel | JX010244 JX010944 JX100104 JX009774 JX010389 |
| *C. acutatum* | ICMP 18668  | Pyrus pyrifolia | Japan | JQ498366 JQ499687 JQ498697 JQ499027 JQ495017 |
| *C. acerbum*  | ICMP 18580  | Malus domestica | China | KF772117 KF772027 KF772087 KF772057 KF772147 |
| *C. acerbum*  | ICMP 12071  | Malus domestica | New Zealand | JX010251 JX010952 JX100108 JX009882 JX010411 |
| *C. acutatum* | ICMP 18621  | Persia americana | New Zealand | JX010246 JX010952 JX009759 JX010386 |
| *C. asiaticum* | ICMP 18683  | Coffea arabica | Thailand | FJ972612 JX009584 JX100853 JX009867 JX010406 |
| *C. boninense* | CBS 12755  | Crinum asiaticum | Japan | JQ005153 JQ005501 JQ005240 JQ005327 JQ005588 |
| *C. cucumae*  | IMI 30482  | Cucurbita sp. | Dominica | JQ498195 JQ499516 JQ498525 JQ498856 JQ499846 |
| *C. fiorinae* | CBS 128417  | Fritonia externa | USA | JQ498292 JQ499613 JQ498622 JQ498953 JQ499493 |
| *C. fiorinae* | CBS 125836  | Malus domestica | USA | JQ498299 JQ499620 JQ498629 JQ498960 JQ499500 |
| *C. fiorinae* | CBS 128517  | Fritonia externa | USA | JQ498299 JQ499613 JQ498622 JQ498953 JQ499493 |
| *C. fiorinae* | CBS 125836  | Malus domestica | USA | JQ498299 JQ499620 JQ498629 JQ498960 JQ499500 |
| *C. fiorinae* | CBS 36503  | Camellia reticulata | China | JQ498339 JQ499660 JQ498669 JQ499000 JQ499590 |
| ATCC 28992   | Malus domestica | USA | JQ498297 JQ499618 JQ498627 JQ498958 JQ499498 |
| *C. fiorinae* | CBS 129938  | Malus domestica | USA | JQ498296 JQ499617 JQ498626 JQ498957 JQ499497 |
| *C. fiorinae* | CBS 129948  | Tulipa sp. | UK | JQ498344 JQ499665 JQ498674 JQ498805 JQ499965 |
| IMI 32496   | Malus pumilla | USA | JQ498301 JQ499622 JQ498631 JQ498962 JQ499592 |
| ATCC 12097  | Rhododendron sp. | USA | JQ498307 JQ499628 JQ498637 JQ498968 JQ499598 |
| CBS 200.35  | Rubus sp. | USA | JQ498293 JQ499614 JQ498623 JQ498954 JQ499944 |
| CBS 490.92  | Solarium lycopersicum | New Zealand | JQ498326 JQ499647 JQ498656 JQ498897 JQ499977 |
| CBS 119293  | Vaccinium corymbosum (blueberry) | New Zealand | JQ498314 JQ499635 JQ498644 JQ498975 JQ499965 |
| *C. fiorinae* | CBS 130416  | Capparis arbores | Thailand | JX010165 JF907426 JX01033 JX009866 JX010405 |
| *C. fiorinae* | CBS 129948  | Malus domestica | China | KF772124 KF772034 KF772094 KF772046 KF772154 |
| *C. fiorinae* | CBS 125836  | Malus domestica | China | KF772125 KF772035 KF772095 KF772046 KF772155 |
| *C. fiorinae* | CBS 129948  | Malus × domestica | China | KF772126 KF772036 KF772096 KF772046 KF772155 |
| *C. fiorinae* | CBS 125836  | Malus × domestica | China | KF791591 KF791570 KF791584 KF791577 KF791598 |
| *C. fiorinae* | CBS 129948  | Malus × domestica | China | KF791591 KF791570 KF791584 KF791577 KF791598 |
| *C. fiorinae* | CBS 129948  | Malus × domestica | China | KF791591 KF791570 KF791584 KF791577 KF791598 |
| *C. fiorinae* | CBS 129948  | Malus × domestica | China | KF791591 KF791570 KF791584 KF791577 KF791598 |
| *C. fiorinae* | CBS 129948  | Malus × domestica | China | KF791591 KF791570 KF791584 KF791577 KF791598 |
| *C. fiorinae* | CBS 129948  | Malus × domestica | China | KF791591 KF791570 KF791584 KF791577 KF791598 |
| *C. fiorinae* | CBS 129948  | Malus × domestica | China | KF791591 KF791570 KF791584 KF791577 KF791598 |
| *C. fiorinae* | CBS 129948  | Malus × domestica | China | KF791591 KF791570 KF791584 KF791577 KF791598 |
| *C. fiorinae* | CBS 129948  | Malus × domestica | China | KF791591 KF791570 KF791584 KF791577 KF791598 |
| *C. fiorinae* | CBS 129948  | Malus × domestica | China | KF791591 KF791570 KF791584 KF791577 KF791598 |
### Table 1. Cont.

| Species Type | Strain | Host | County |
|--------------|--------|------|--------|
| *C. gloeosporioides* | CBS 112999 | Citrus sinensis | Italy |
| | CBS 119204 | Musa |ollandia |
| | F11PGQX17 | *M. x domestica* | China |
| | F12PGQX01 | *M. x domestica* | China |
| | F12PGQX30 | *M. x domestica* | China |
| | F12PGQX33 | *M. x domestica* | China |
| | F12PGQX34 | *M. x domestica* | China |
| | F11PGQX23 | *M. x domestica* | China |
| *C. godetiae* | CBS 153.44 | *Clarkia hybrida* | Denmark |
| | CBS 198.53 | *M. sylvestris* | Netherlands |
| | ICMP 10492 | *C. horticola* | Japan |
| | CBS 126524 | Coffea | Brazil |
| | CBS 124949 | *Theobroma cacao* | Panama |
| | ICMP 18672 | *Litchi chinensis* | Japan |

**GenBank No.**

| Species Type | Strain | Host | County |
|--------------|--------|------|--------|
| *C. gloeosporioides* | CBS 112999 | Citrus sinensis | Italy |
| | CBS 119204 | Musa |ollandia |
| | F11PGQX17 | *M. x domestica* | China |
| | F12PGQX01 | *M. x domestica* | China |
| | F12PGQX30 | *M. x domestica* | China |
| | F12PGQX33 | *M. x domestica* | China |
| | F12PGQX34 | *M. x domestica* | China |
| | F11PGQX23 | *M. x domestica* | China |
| *C. godetiae* | CBS 153.44 | *Clarkia hybrida* | Denmark |
| | CBS 198.53 | *M. sylvestris* | Netherlands |
| | ICMP 10492 | *C. horticola* | Japan |
| | CBS 126524 | Coffea | Brazil |
| | CBS 124949 | *Theobroma cacao* | Panama |
| | ICMP 18672 | *Litchi chinensis* | Japan |

1. Cannon et al. (2012) [10]; 2. Damm et al. (2012a) [12]; 3. Weir et al. (2012) [52]; 4. Damm et al. (2012b) [53].

### 3. Results

#### 3.1. Isolate Isolation

In total, 151 isolates were isolated from symptomatic leaf and fruit lesions in four apple-growing provinces. Among these, 98 were from bitter rot lesions, and 53 were from GLS lesions. Based on the conidial morphology and ITS sequence, 17 isolates were typical for the *C. acutatum* complex, while 134 isolates were typical for the *C. gloeosporioides* complex.

#### 3.2. Phylogenetic Analysis

Based on ITS sequences and cultural characters, 32 representative isolates were chosen for further phylogenetic analysis. The five-locus (ITS, ACT, GAPDH, CHS-1 and TUB2) phylogenetic analysis included 51 reference isolates [10,12,27]. Concatenated sequence
alignment contained a total of 1916 characters, among which 551 were parsimony informative (28.8%). The BI tree, along with both the Bayesian posterior probability values and maximum likelihood bootstrap support values, are shown in Figure 1. The Bayesian tree was identical to the maximum likelihood tree in topology.

The phylogram supported eight defined clades, representing *C. aenigma*, *C. alienum*, *C. fructicola*, *C. gloeosporioides*, *C. nymphaeae*, *C. siamense* and a candidate for a new species, respectively. Four isolates clustered with *C. hymenocallidis* (CBS 125378), and one isolate grouped with *C. siamense sensu stricto* (CBS 130417 and ICMP 17795), which both belong to *C. siamense sensu lato* [52]. The clades of *C. fructicola* (CBS 130416), *C. aenigma* (ICMP 18608 and, ICMP 18686), *C. alienum* (ICMP 12071 and ICMP 18621) and *C. gloeosporioides* (CBS 112999 and CBS 119204) each included nine, three, one and six apple isolates, respectively.

The remaining eight isolates from the diseased apple fruits belonged to the *C. acutatum* complex. One isolate clustered together with *C. nymphaeae* (CBS 515.78 and IMI 370491), while the other seven formed a separate clade together with CBS 128555 (Figure 1). As revealed by the previous multi-locus molecular phylogenetic analysis [12], *C. fioriniae* contains two well-separated clades; one clade contains CBS 128555, and the other clade contains the type strain CBS 128517. Here, we propose that the CBS 12855 clade should better be defined as an independent taxon unit, which we have named *C. orientalis*. The new species delimitation was also supported by the PHI analysis in which no obvious evidence of recombination was detected between the two clades (Figure 2).

![Figure 2](image_url)

**Figure 2.** Pairwise homoplasy index (PHI) test of *C. fioriniae* and *C. orientalis* using both LogDet transformed and splits decomposition. PHI test results (\(\Phi_W\) > 0.05) indicate the lack of recombination within the dataset.

### 3.3. Taxonomy

Based on the result of multigene phylogeny, the 32 *Colletotrichum* isolates characterized in this study were grouped into seven species: *C. aenigma* (three isolates), *C. alienum* (one isolate), *C. fructicola* (nine isolates), *C. gloeosporioides* (six isolates), *C. nymphaeae* (one isolate), *C. siamense* (five isolates) and *C. orientalis* (seven isolates).

**Colletotrichum aenigma** B. Weir & P.R. Johnst. Studies in Mycology 73: 135. 2012. [52] Figure 3(A1–A5).
Figure 3. Morphological and cultural characters of Colletotrichum isolates: (A) C. aenigma; (B) C. alienum; (C) C. fructicola; (D) C. gloeosporioides; (E) C. nymphaeae; (F) C. siamense; (G) C. hymenocallidis. Upper (1) and reverse (2) of cultures on PDA; (3) conidiophores; (4) conidia; (5) appressoria. Bars = 10 μm.

Description: Vegetative hyphae are 1–5.5 μm diam, hyaline, smooth-walled, septate and branched. Conidiophores are formed directly on hyphae. Conidiophores are hyaline and smooth-walled; they are sometimes septate and branched. Conidiogenous cells are hyaline, smooth-walled, cylindrical and not clearly separated from the hyphae by a septum. Conidia are straight, cylindrical or clavate with rounded ends; sometimes they taper slightly to one end, $(11.8–)15.5–17.5(–18.8) \times (3.8–)4.5–5.5(–6) \, \mu m$, mean ± SD = $16.46 \pm 1.30 \times 5.06 \pm 0.56 \, \mu m$ (n = 50), L/W ratio = 3.3. Appressoria are elliptical or ovoid; some have broad, irregular
Conidiogenous cells are hyaline, smooth, ovoid-elliptical or short-cylindrical and often clearly have a septum. Conidia are hyaline, smooth-walled, septate and branched.

Notes: The ITS sequence analysis could not separate it from some of the isolates of C. aenigma by ITS sequence analysis, nor from C. tropicale by ACT. The conidia of the holotype (ICMP 18608) of C. aenigma were (12-)14–15(–16.5) × (5-)6–6.5(–7.5) µm, and the appressoria were subglobose [52], whereas the isolate F12PGXY03 had longer and thinner conidia, and the appressoria were generally oval-shaped and longer than those of ICMP 18608. Additionally, the cultural characters of our isolates were different from those of ICMP 18608.

Colletotrichum alienum B. Weir & P.R. Johnst. Studies in Mycology 73: 139. 2012. [52] Figure 3(B1–B5).

Description: Vegetative hyphae are 1–9 µm diam, hyaline, smooth-walled, septate and branched. Conidiophores are hyaline, smooth-walled, septate and branched. Conidiogenous cells are hyaline, smooth, ovoid-elliptical or short-cylindrical and often clearly have a septum. Conidia are straight, mostly cylindrical with broadly rounded ends; a few taper towards the basal end, (12.9–)15–17(–19.7) × (3.4–)4–5(–6.1) µm, mean ± SD = 16.27 ± 1.37 × 4.73 ± 0.56 µm (n = 50), L/W ratio = 3.4. Appressoria are mostly simple and subglobose or elliptical; a few have broad, irregular lobes, (6.8–)8–10(–10.8) × (5.1–)6–7(–7.6) µm, mean ± SD = 8.91 ± 0.98 × 6.46 ± 0.57 µm (n = 50), L/W ratio = 1.4. Colonies grown on the PDA (Difco) are 85 mm after 7 d at 25 °C; the aerial mycelium is dense, cottony and gray with an orange conidial ooze visible towards the center; in reverse, it is dark gray towards the center with sporadic black flecks and pale gray towards the edge.

Specimen examined: China, Henan Province: Zhengzhou City, on the fruit surface of an apple (Malus × domestica Borkh.), 28 September 2011, Dandan Fu F11PGZH02.

Colletotrichum fructicola Prihastuti, L. Cai & K.D. Hyde. Fungal Diversity 39: 96, 2009. [54] Figure 3(C1–C5).

Description: Vegetative hyphae are 1–11 µm diam, hyaline to pale brown, smooth-walled, septate and branched. Conidiophores are hyaline and smooth-walled, septate and branched. Conidiogenous cells are hyaline, smooth-walled, septate and branched. Conidia are hyaline, smooth-walled, cylindric and not clearly separated from the hyphae by a septum. Conidia are hyaline, asperate, straight and cylindrical with both ends rounded or one end slightly acute, (13.1–)14.5–16(–18.5) × (4.5–)5–5.5(–6.2) µm, mean ± SD = 15.38 ± 1.16 × 5.29 ± 0.40 µm (n = 50), L/W ratio = 2.9. Appressoria are single or in loose groups, pale to dark brown, ovoid, cylindrical or fusoid and sometimes slightly irregular, (6–)8.5–11(–13) × (4.4–)5.5–6.5(–8.4) µm, mean ± SD = 9.66 ± 1.74 × 5.94 ± 0.81 µm (n = 50), L/W ratio = 1.6. Colonies on the PDA are 78–80 mm after 7 d. The aerial mycelium is white to pale gray, dense and cotonny; in reverse, it is dark gray towards the center and pale gray at the edge.

Specimen examined: China, Henan Province: Shangqiu City, on the fruit surface of an apple (Malus × domestica Borkh.), 6 September 2012, Dandan Fu F12PGSQ01, F12PGSQ05; from the leaf of an apple, Wei Wang, WW12PGYSQ06; Xiayi County, 7 September 2012, F12PGXY01. Liaoning Province: Xingcheng City, F10PGCJJ1, F10PGCJJ3; Huiluado City, F10PGHLD1; Shandong Province: Yantai City, 29 September 2011, Dandan Fu, F11PGYT02, F11PGYT04.

Notes: The ITS sequence analysis did not separate C. fructicola from C. aescynomenes, and the ACT sequence analysis could not separate it from C. alienum, C. diansei, C. queenslandicum and C. siamense. Similarly, neither GAPDH nor TUB2 separated this species from C. alienum. The CHS-1 sequence analysis did not separate it from some of the isolates of C. siamense. Nevertheless, these taxa were well-distinguished using multi-gene analysis.
Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. Atti Reale Ist. Veneto Sci. Lett. Arti., Series 6, 2: 670. 1884. [55] Figure 3(D1–D5).

Description: Vegetative hyaline are 1–8 μm diam, hyaline to medium brown, smooth, septate and branched. Conidiophores are hyaline, smooth-walled, one to three celled and sometimes branched. Conidiogenous cells are hyaline, smooth, cylindrical and often clearly have a septum. Conidia are straight and mostly cylindrical with broadly rounded ends; they are sometimes slightly acute, tapering gradually to the ends, (13.1–)14–15(–16.1) × (3.8–)4.5–5.5(–5.8) μm, mean ± SD = 14.48 ± 0.70 × 5.12 ± 0.41 μm (n = 50), L/W ratio = 1.4. Appressoria are simple or in small groups and subglobose or elliptical; a few are irregular, (6.6–)7.5–9(–13.8) × (4.6–)5.5–6(–7.2) μm, mean ± SD = 8.32 ± 1.24 × 6.02 ± 0.72 μm (n = 50), L/W ratio = 1.4. Colonies grown on the PDA(Difco) are 75–80 mm after 7 d at 25 °C; the aerial mycelium is dense, cottony and pale gray to medium gray towards the center, in reverse, olivaceous gray, with sporadic dark gray flecks. Colonies on the OA are flat with an entire margin; the aerial mycelium is sparse, panniform and pale gray. An orange conidial ooze is visible in the mycelium.

Specimen examined: China, Shaanxi Province: Qian County, on the fruit surface of an apple (Malus × domestica Borkh.), 24 September 2011, Dandan Fu F11PGQX17, 20 September 2010, F12PGLQ30, F12PGLQ33, F12PGLQ34; Dali County, 16 August 2012, F12PGDL01; Henan Province: Zhengzhou City, 28 September 2011, F11PGZH23.

Colletotrichum nymphaeae (Pass.) Aa. Netherlands J. Pl. Pathol., Supplement 1 84: 110. 1978. [56] Figure 3(E1–E5).

Description: Vegetative hyphae are 1–5 μm diam, hyaline, smooth, septate and branched. Conidiophores are formed directly on hyphae. Conidiophores are hyaline and smooth; a few are septate and branched. Conidiogenous cells are hyaline, smooth, cylindrical or fusiform and not clearly separated from the hyphae by a septum. Conidia are straight and cylindrical to clavate, with one end rounded and the other end or two ends acute, (6.8–)9–13(–15.9) × (3.4–)4–4.5(–5.3) μm, mean ± SD = 11.24 ± 2.19 × 4.24 ± 0.44 μm (n = 50), L/W ratio = 2.7. Appressoria are simple or in a small group and mostly subglobose or elliptical; a few have an irregular outline, (4.8–)6.5–7.5(–9) × (4.3–)5–6(–7.9) μm, mean ± SD = 6.97 ± 0.85 × 5.64 ± 0.60 μm (n = 50), L/W ratio = 1.2. Colonies on the PDA are flat with an entire margin. The aerial mycelium is sparse, grayish-yellow or cinnamon towards the center and white at the edge; in reverse, it is dark olivaceous gray. It has a growth rate of 54–60 mm after 7 d.

Specimen examined: China, Liaoning Province: Zhuanghe City, on the fruit surface of an apple (Malus × domestica Borkh.), 24 September 2011, Dandan Fu F11PGBYS12.

Colletotrichum siamense Prihastuti, L. Cai & K. D. Hyde. Fungal Diversity 39: 98. 2009. [54] Figure 3(F1–F5,G1–G5).

Description: Vegetative hyphae are 1–8 μm diam, hyaline to pale brown, smooth-walled, septate and branched. Conidiophores are hyaline to pale brown, smooth-walled and one or two celled; a few are branched. Conidiogenous cells are hyaline, smooth, cylindrical and clearly separated from the hyphae by a septum. Conidia are hyaline, asceptate, straight and cylindrical with both ends rounded, (11.9–)14.5–15.5(–18.9) × (4–)4.5–5(–5.5) μm, mean ± SD = 15.08 ± 1.14 × 4.87 ± 0.31 μm (n = 50), L/W ratio = 3.1. Appressoria are single or in loose groups, pale to dark brown, ovoid and subglobose or short, mean ± SD = 9.79 ± 1.60 × 5.94 ± 0.77 μm (n = 50), L/W ratio = 1.6. Colonies on the PDA have an entire margin. The aerial mycelium is white, and the colonies are dense, cotty, white to pale gray or dark gray. Occasionally, orange conidial ooze is visible in the center; in reverse, it is buff with sporadic dark gray spots or greyish dark towards the center and pale gray at the edge. Colonies on the PDA are 68–75 mm after 7 d.

Specimen examined: China, Henan Province: Shangqiu City, 6 September 2012, Dandan Fu F12PGSQ02; Mengjin County, 12 August 2012, Dandan Fu, F12PGMJ01; Shaanxi Province: Liquan County, on the fruit surface of an apple, 24 September 2011, Dandan Fu,
Notes: After Prihastuti et al. separated *Colletotrichum siamense* from the *C. gloeosporioides* complex, more isolates from multiple hosts were identified as this species [54]. ITS sequences separated *C. siamense* well from other taxa, but the ACT sequence did not separate it from *C. alienum*, *C. hymenocallidis*, *C. queenslandicum* or *C. fructicola*. Similarly, GAPDH and TUB2 do not separate this species from *C. hymenocallidis*. *C. hymenocallidis* was first introduced by Yang et al. from *Hymenocallis americana* [57] but was recently synonymized with *C. siamense* [52]. However, Sharma et al. considered *C. siamense* to be a species complex based on an ApMat sequence analysis because *C. siamense* showed high sequence variability [58]. Moreover, Liu et al. indicated that more isolates need to be included to support further splitting of *C. siamense*, which possibly resurrects *C. hymenocallidis* [59].

**Colletotrichum orientalis** Dandan Fu & G.Y. Sun, sp. nov. Figure 4.

![Figure 4. Colletotrichum orientalis (F10PGBYS08): (A–D) conidiophores; (E,F) conidia; (G–I) appressoria; (J) apple fruit lesion symptom with non-wounded conidial inoculation. Scale bars = 10 μm. (K,L) Colony on PDA (F10PGBYS08); (M,N) colony on PDA (F10PGBYS04); (O,P) colony on PDA (F10PGBYS05).](image-url)
Mycobank: MB 808171.

Etymology: Referring to the isolates collected from the eastern region of China.

Description: Vegetative hyphae are 1–6 µm, hyaline, smooth-walled, septate and branched. Conidiophores are formed directly on the hyphae. Conidiophores are hyaline, smooth-walled, simple or septate and branched. Conidia are hyaline, smooth-walled, aseptate, straight and fusiform or cylindrical with both ends acute, (12.8–)14–16(–18.5) × (3.9–)4–5(–5.5) µm, mean ± SD = 15.07 ± 1.23 × 4.51 ± 0.38 µm (n = 50), L/W ratio = 3.3 µm. Appressoria are single or in loose groups, pale-to-medium brown, smooth, oval-shaped and ellipsoidal or irregularly outlined, (7–)8–9.5(–11.5) × (4.4–)5.5–6(–7.2) µm, mean ± SD = 8.74 ± 0.99 × 5.84 ± 0.53 µm (n = 50), L/W ratio = 1.5. Colonies on the PDA have an entire margin and are compacted cottony to felty. They are orangish red towards the center and pale gray towards the edge. The aerial mycelium is white to pale gray, and the conidiomata are sparse with masses of orange conidia. In reverse pale brownish pink. Colonies on the OA have an entire margin. The aerial mycelium is sparse, white to pale gray, and on the surface with visible masses of orange conidia scattered in circles; in reverse, it is pale buff. Colonies on the PDA are 45–51 mm after 7 d (67–75 mm in 10 d).

Holotype: China, Liaoning Province: Zhuanghe City, on the fruit surface of an apple (Malus × domestica Borkh.), 20 September 2010, Coll. Jieli Zhuang, F10PGBYS08 (CGMCC3.17216; isotype in HMAS244986 as dry culture).

Additional specimen examined: China, Liaoning Province: Zhuanghe City, on the fruit surface of an apple (Malus × domestica Borkh.), 20 September 2010, Jieli Zhuang, F10PGBYS01 (CGMCC 3.17217), F10PGBYS02–04, F10PGBYS07–08, F10PGBYS10.

Notes: Freeman and Shabi studied isolates from fruit rot of apples and peaches (based on the ITS sequence, probably identifiable as C. fioriniae), which produced lesions on many different fruits, indicating that isolates of this group have the ability to cross-infect fruit from multiple hosts [60]. In this paper, we isolated seven isolates from apple bitter rot in Liaoning Province. A phylogenetic analysis (Figure 1) showed that they constituted a monophyletic clade together with the six C. fioriniae isolates (CBS 129938, CBS200.35, ATCC 28992, CBS 119293, CBS 128555 and CBS 490.92). In Damm et al. [12], the clade was well-separated from the clade containing the C. fioriniae holotype CBS 128517. Separation between the two clades was also evident in Damm et al. [12], which was treated as intraspecific heterogeneity. In this study, the PHI analysis detected no significant evidence of recombination between the two clades (Figure 2). Therefore, we denominate the clade containing the ABR isolates a new species.

3.4. Pathogenicity Tests

In the fruit infection assays, the isolates isolated from apples with bitter rot symptoms were pathogenic to the apple fruits in both the unwounded and wounded inoculations (Table 2). Dark brown rot lesions, similar in appearance, were produced in all cases (Figure 5). Of the non-wounded inoculations, Colletotrichum alienum (F11PGZH02) had the highest infection incidence (100%), whereas the isolates of C. gloeosporioides (F11PGQX17) and C. orientalis (F10PGBYS08) had the lowest incidences (33%); the others were in the middle. Of the wounded inoculations, all isolates had a 100% infection incidence. Lesions incurred by different isolates were similar in size, except that the lesions incurred by C. nymphaeae (F10PGBYS12, belonging to the C. acutatum complex) were apparently smaller (Figure 5).
Table 2. Pathogenicity test of representative Colletotrichum isolates on Fuji apple fruits.

| Species         | Isolate    | Non-Wounded | Wounded |
|-----------------|------------|-------------|---------|
| C. alienum      | F11PGZH02  | +++         | +++     |
| C. fructicola   | F12PGSQ01  | ++          | +++     |
| C. gloeosporioides | F11PGQX17 | +           | +++     |
| C. nymphaeae    | F10PGBYS12 | ++          | +++     |
| C. siamense     | F12PGSQ02  | ++          | +++     |
| C. orientalis   | F10PGBYS08 | +           | +++     |

+: Infection incidence < 50%; ++: 50% < Infection incidence < 100%; +++: Infection incidence = 100%.

Figure 5. Typical field symptoms of ABR and GLS diseases (top) and artificial inoculation results (bottom). Top, field symptoms, (A1–A3) represent fruit ABR, GLS on leaves and fruits, respectively. Bottom, (1A–8B) represent typical symptoms on Fuji apples under unwounded or wounded inoculation conditions. A: Non-wounded; B: wounded; 1: C. acerina (F12PGXY03); 2: C. alienum (F11PGZH02); 3: C. fructicola (F12PGSQ01); 4: C. gloeosporioides (F11PGQX17); 5: C. siamense (F12PGSQ02); 6: C. siamense (F11PGLQ22); 7: C. nymphaeae (F10PGBYS12); 8: C. orientalis (F10PGBYS08). (9A–9C) Symptoms on cv. Gala apple leaves and fruits inoculated with conidial suspension of isolate C. fructicola W12PGYSQ06 from GLS. (9A) Leaf inoculation; (9B) fruit from unwounded inoculation; (9C) fruit from wounded inoculation.
Isolates isolated from the GLS lesions caused GLS lesions on both the apple fruits and leaves (Table 3). The isolates of *C. aenigma* (F12PGXY03, W12PGYXY15) and *C. fructicola* (F12PGSQ05, W12PGYSQ06) were pathogenic on the leaves and fruits of Gala apples, but non-pathogenic on Fuji apple leaves or fruits in the non-wounded inoculation (Figure 5), which is in accordance with the observation that Fuji apples are resistant to GLS disease [61].

Table 3. Pathogenicity test of selected isolates on apple leaves.

| Species          | Isolate    | Origin | Inoculation Cultivar | Inoculation Outcome |
|------------------|------------|--------|----------------------|---------------------|
| *C. aenigma*     | F12PGXY03  | GLS lesion | Fuji                 | −                   |
|                  | W12PGYXY15 | GLS lesion | Gala                 | +                   |
| *C. fructicola*  | F12PGSQ05  | GLS lesion | Fuji                 | −                   |
|                  | W12PGYSQ06 | GLS lesion | Gala                 | +                   |
| *C. alienum*     | F11PGZH02  | ABR lesion | Fuji                 | −                   |
| *C. fructicola*  | F12PGSQ01  | ABR lesion | Fuji                 | −                   |
| *C. gloeosporioides* | F11PGQX17  | ABR lesion | Fuji                 | −                   |
| *C. nymphaeae*   | F10PGBYS12 | ABR lesion | Fuji                 | −                   |
| *C. siamense*    | F12PGSQ02  | ABR lesion | Fuji                 | −                   |
| *C. orientalis*  | F10PGBYS08 | ABR lesion | Fuji                 | −                   |

+: Pathogenic; −: Non-pathogenic.

4. Discussion

China is the largest apple-producing country in the world. Bitter rot has been a common disease in almost all apple production areas and can cause large economic losses under disease-favorable temperature and humidity conditions. Glomerella leaf spot (GLS) has been a severe foliar disease on cvs. Gala, Jonagold and Golden Delicious in the USA and Brazil. It was found first in Henan, China, in 2010 [28]. Now, it has become prevalent in all major apple-producing areas in China. Thus far, however, the species diversity of apple *Colletotrichum* pathogens in China is largely unclear. In this study, we collected and characterized 151 isolates from four apple-producing provinces and identified six known species, as well as one new species, demonstrating that diverse *Colletotrichum* species can infect apples. Moreover, *C. orientalis* was shown for the first time to be an apple *Colletotrichum* pathogen.

Among the identified species, *C. siamense, C. fructicola, C. aenigma, C. alienum* and *C. gloeosporioides* belong to the CGSC, while *C. orientalis* and *C. nymphaeae* belong to the CASC. Overall, the CGSC species appear to be more prevalent compared with the CASC species. Moreover, fruit isolates and leaf isolates differ significantly in their genetic makeups. Seven species were recognized as isolates from apple fruits, whereas only two (*C. fructicola* and *C. aenigma*) were recognized as leaf spot isolates. In a pathogenicity test, ABR isolates fail to incur GLS symptoms, and the GLS isolates fail to incur ABR symptoms, indicating a pathogenic differentiation among the two groups of pathogens. Such results are in accordance with a previous study that demonstrated the intraspecific differentiation in the pathogenicity of GLS and ABR for *C. fructicola* [62].

*C. siamense* is a species that includes members from diverse hosts and that has a worldwide distribution. The diversity is so high that there has been controversy regarding whether it should be treated as a single species or a species complex. In a recent study
carried out by Liu and others [63], six independent species very close to C. siamense s. str. (C. communis, C. hymenocallidis, C. dianesei, C. endomangiferae, C. jasmini-sambac and C. murrayae) were renamed as C. siamense. In this study, the four characterized isolates clustered together with C. hymenocallidis, and one isolate clustered with C. siamense s. str. Based on broad species criteria, these isolates should all be regarded as C. siamense sensu lato. C. fructicola represents another important pathogen species identified in this study. C. fructicola has a very broad host range, having been isolated from over eight plant families as endophytes and as plant pathogens. In this study, C. fructicola was isolated from both bitter rot and Glomerella leaf spot lesions. C. fructicola causes Glomerella leaf spot in Brazil but has been more commonly identified as a bitter rot pathogen in central USA, Brazil and Uruguay. In Uruguay in particular, most isolates from apple bitter rot were identified as C. fructicola. Interestingly, despite the prevalence of C. fructicola in Uruguay, Glomerella leaf spot disease does not occur in the field [62]. In the future, it would be interesting to determine whether there are distinctive C. fructicola populations for isolates from leaf lesions and fruit lesions in China.

In a previous study [12], C. fiorinae has been defined as a species with two well-separated clades. We propose here that the two clades should be regarded as different species due to the fact that the pairwise homoplasy index (PHI) analysis in SplitsTree did not detect evidence of recombination between them. Therefore, we have named the new lineage C. orientalis. C. alienum and C. gloeosporioides are two other species common in fruits. Interestingly, C. alienum has only been reported in New Zealand and Australia, and C. gloeosporioides has never been reported on apples in China. The identification of these species on apples in China highlights the importance of a diversity survey.

Compared with apple bitter rot, GLS is a relatively new disease. Velho and others have suggested that GLS pathogens originate from apple bitter rot pathogens [62]. In this study, all isolates caused fruit rot upon wound inoculation, whereas only the isolates from the leaf spot lesions incurred leaf spot symptoms. Importantly, such pathogenic differentiation could occur in the same species (e.g., F12PGSQ01 and W12PGYSQ06). Such a pathogenicity differentiation pattern is in line with the report by Velho and others [62]. Isolates showing an intraspecific pathogenic variation would be valuable resources for comparative studies aiming to dissect the mechanisms that underlie the adaptive evolution of apple Colletotrichum pathogens.

In summary, based on a systemic survey of Colletotrichum isolates, in this study, we identified seven species associated with the GLS and ABR diseases in China, highlighting the rich species diversity of Colletotrichum spp. on apples. There are tens of apple-producing provinces in China that are variable in terms of climate and soil conditions; further survey efforts into the hidden species diversity and the structural variations among the different regions is critical for effective control of these two important diseases.

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