Morphological and molecular phylogenetic analyses reveal three species of Colletotrichum in Shandong province, China

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Abstract
Colletotrichum has numerous host range and distribution. Its species are important plant pathogens, endophytes and saprobes. Colletotrichum can cause regular or irregular depressions and necrotic lesions in the epidermal tissues of plants. During this research Colletotrichum specimens were collected from Mengyin County, Shandong Province, China. A multi-locus phylogenetic analysis of ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS sequence data combined with morphology, revealed a new species and two known species, viz. C. mengyinense sp. nov., C. gloeosporioides and C. pandanicola, belonging to the C. gloeosporioides species complex. The new species is described and illustrated in this paper and compared with taxa in the C. gloeosporioides species complex.

Keywords
Colletotrichum, Glomerellaceae, multi-gene phylogeny, new species, taxonomy

Introduction

Colletotrichum species (Glomerellaceae, Glomerellales) is one of the ten economically most important fungal plant pathogens worldwide (Dean et al. 2012). It was first observed by Tode (1790), who divided it into Vermicularia. Corda (1831) established Colletotrichum based on the characteristic of the conidiomata with setae in Vermicularia.
Colletotrichum is based on the type species Colletotrichum lineola which was associated with a member of the Apiaceae (Jayawardena et al. 2017). The sexual morph belongs to Glomerella. The asexual morph is characterized by acervuli born in the skin of the host, often producing brown sharp setae, colorless or brown conidiophores with separate, conidia colorless, pseudomonas, cylindrical or crescent-shaped (Damm et al. 2009).

Currently, more than 900 epithets of Colletotrichum are listed in Index Fungorum (http://www.indexfungorum.org/; accessed 22 November 2021). Colletotrichum has been studied for more than 200 years and the classification of Colletotrichum has undergone major changes (Jayawardena et al. 2016). In order to clarify its complex nature, the species are classified into 14 species complexes (Bhunjun et al. 2021). Specifically, C. gloeosporioides has been considered as a complex species for a long time.

The name C. gloeosporioides was first proposed by Penzig based on Vermicularia gloeosporioides which was collected from Citrus in Italy (Weir et al. 2012). Early in the study of C. gloeosporioides species complex, taxonomic concepts used were based on apparent features such as morphological characters, host species, size and shape of conidia and appressoria, presence or absence of setae, aspect, color and growth rate in culture, whether or not the teleomorph develops, etc (Weir et al. 2012). Nonetheless, Sutton commented that “no progress in the systematics and identification of isolates belonging to this complex is likely to be made based on morphology alone”. Fortunately, with the development of molecular systematics, gene method is applied to taxonomy of Colletotrichum complexes. Multi-gene phylogeny analysis is of great significance to the study of the classification of C. gloeosporioides species complex and related concepts of species (Cannon et al. 2012; Damm et al. 2012; Weir et al. 2012).

The aim of this study was to explore the diversity of Colletotrichum species from symptomatic leaves and diseased fruit of plants in Shandong Province, China. We present a new species and two known species, C. mengyinense sp. nov., C. gloeosporioides and C. pandanicola based on phylogenetic data and morphology.

**Materials and methods**

**Isolation and morphological studies**

The samples were collected from Mengyin County, Shandong Province, China. The strains of Colletotrichum were isolated from symptomatic leaves of Rosa chinensis and diseased fruit of Juglans regia using single spore and tissue isolation methods (Chomnunti et al. 2014). The spore suspension was obtained and spread onto PDA plate and incubated for one day under the biochemical incubator. After germination, the spores were transferred to a new PDA plate to obtain pure culture. Additionally, the surface sterilized plant tissue isolation was used to obtain sterile isolates from the host plant. About 25 mm² tissue fragments were taken from the margin of tissue lesions and
surface sterilized by consecutively immersing in 75% ethanol solution for 60 s, 5% sodium hypochlorite solution for 30 s, and then rinsed in sterile distilled water for 60 s (Gao et al. 2013; Liu et al. 2015). The surface sterilized plant tissue was dried with sterilized paper and moved on the PDA plate (Cai et al. 2009). All the PDA plates were incubated at biochemical incubator at 25 °C for 3–4 days, then hyphae were picked out of the periphery of the colonies and inoculated on to new PDA plates.

Following 5–14 days of incubation, morphological characters were recorded (Cai et al. 2009). Photographs of the colonies were taken at 7 days and 14 days using a digital camera (Canon G7X). Micromorphological characters of colonies were observed using stereomicroscope (Olympus SZX10) and microscope (Olympus BX53), both fitted with high definition color digital cameras to photo document conidia and so on of fungal structures. All *Colletotrichum* strains were stored in 10% sterilized glycerin and sterile water at 4 °C for deep studies in the future. Every specimen was deposited in the Herbarium of the Department of Plant Pathology, Shandong Agricultural University (HSAUP). Living cultures were deposited in the Shandong Agricultural University Culture Collection (SAUCC). Taxonomic information of the new taxa was submitted to MycoBank (http://www.mycobank.org).

**DNA extraction and amplification**

Genomic DNA was extracted from *Colletotrichum* fungal mycelia grown on PDA after 5–7 days, using a modified cetyltrimethylammonium bromide (CTAB) buffer, and then it was incubated at 65 °C for 30 min with occasional gentle inverting (Guo et al. 2000). Gene sequences were obtained from seven genes loci including the internal transcribed spacer regions with intervening 5.8S nrRNA gene (ITS), partial glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), partial chitin synthase 1 gene (CHS-1), partial actin gene (ACT), partial beta-tubulin gene (TUB2), partial calmodulin gene (CAL) and partial glutamine synthetase gene (GS) were amplified and sequenced using primers pairs (Table 1).

PCR was performed using an Eppendorf Master Thermocycler (Hamburg, Germany). Amplification reactions were performed in a 25 μL reaction volume which contained 12.5 μL 2× Taq Plus Master Mix II (Vazyme, Nanjing, China), 1 μL of each forward and reverse primer (10 μM) (Tsingke, Qingdao, China), and 1 μL template genomic DNA in amplifier, and were adjusted with distilled deionized water to a total volume of 25 μL. PCR parameters were as follows: 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at a suitable temperature for 30 s, extension at 72 °C for 1 min and a final elongation step at 72 °C for 10 min. The annealing temperature for each gene was 52 °C for ITS and GS, 59 °C for CAL, 60 °C for GAPDH, 58 °C for ACT and CHS-1, 55 °C for TUB2. The PCR products were visualized on 1% agarose electrophoresis gel. Sequencing was conducted by the Tsingke Company Limited (Qingdao, China) bi-directionally. Consensus sequences were obtained using MEGA 7.0 (Kumar et al. 2016). All sequences generated in this study were deposited in GenBank (Table 2).
Phylogenetic analyses

Novel sequences were generated from the nine strains in this study, and all reference available sequences of *Colletotrichum* species were downloaded from GenBank. Multiple sequence alignments for ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS were constructed and carried out using the MAFFT v.7.11 online programme (http://mafft.cbrc.jp/alignment/server/, Katoh et al. 2019) with the default settings, and manually corrected where necessary. To establish the identity of the isolates at species level, phylogenetic analyses were conducted individually for each locus and then as combined analyses of seven loci (ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS). Phylogenetic analyses were based on maximum likelihood (ML) and Bayesian.

Inference (BI) for the multi-locus analyses. For BI, the best evolutionary model for each partition was determined using MrModeltest v. 2.3 (Nylander 2004) and incorporated into the analyses. ML and BI were run on the CIPRES Science Gateway portal (https://www.phylo.org/) using RaxML-HPC2 on XSEDE (8.2.12) (Miller et al. 2012; Stamatakis 2014) and MrBayes on XSEDE (3.2.7a), respectively (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; Ronquist et al. 2012). For ML analyses the default parameters were used and BI was carried out using the rapid bootstrapping algorithm with the automatic halt option. Bayesian analyses included seven parallel runs of 5,000,000 generations, with the stop rule option and a sampling frequency of 1000 generations. The burn-in fraction was set to 0.25 and posterior probabilities (PP) were determined from the remaining trees. The resulting trees were plotted using FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and edited with Adobe Illustrator CS6.0. New sequences generated in this study were deposited at GenBank (https://www.ncbi.nlm.nih.gov; Table 2).

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**Table 1.** Gene regions and respective primer pairs used in the study.

| Locus                              | Gene                        | Primer     | Direction | Sequence (5’-3’)                  |
|------------------------------------|-----------------------------|------------|-----------|-----------------------------------|
| The internal transcribed spacer regions with intervening 5.8S rRNA gene | ITS                         | ITS5       | Forward | GGA AGT AAA AGT CGT AAC AAG G     |
|                                    |                             | ITS4       | Reverse   | TCC TCC GGT TAT TGA TAT GC        |
| Partial glyceraldehyde-3-phosphate dehydrogenase gene | GAPDH                      | GDF1       | Forward   | GCC GTC AAC GAC CCC TTC ATT GA    |
|                                    |                             | GDR1       | Reverse   | GGG TGG AGT CGT ACT TGA GCA TGT   |
| Partial chitin synthase 1 gene     | CHS-1                       | CHS-79F    | Forward   | TGG GGC AAG GAT GCT TGG AAG AAG   |
|                                    |                             | CHS-354R   | Reverse   | TGG AAG AAC CAT CTG TGA GAG TGG   |
| Partial actin gene                 | ACT                         | ACT-512F   | Forward   | ATG TGC AAG GCC GGTT TTC GC       |
|                                    |                             | ACT-783R   | Reverse   | TAC GAG TCC TTC TGG CCC AT        |
| Partial beta-tubulin gene          | TUB2                        | Br-2a      | Forward   | GGT AAC CA ATC GGT GCT GCT TTC    |
|                                    |                             | Br-2b      | Reverse   | ACC CTC AGT GTA GTG ACC CTT GGC   |
| Partial calmodulin gene            | CAL                         | CL1        | Forward   | GAR TWC AAG GAG GCC TTC TC        |
|                                    |                             | CL2A       | Reverse   | TTT TGG CAT CAT GAG TTG GAC       |
|                                    |                             | CL1C       | Forward   | GAA TTC AAG GAG GCC TTC TC        |
|                                    |                             | CL2C       | Reverse   | CTT CTG CAT CAT GAG CTG GAC       |
| Partial glutamine synthetase gene  | GS                          | GSLF3      | Forward   | GAT ACG CCT CCT CCA GCG TT        |
|                                    |                             | GSLR1      | Reverse   | AGR CGC ACA TGG TCA GTA TCG       |
| Species                  | Strain/Isolate       | Host/Substrate         | GenBank accession number |
|-------------------------|----------------------|------------------------|-------------------------|
| **Colletotrichum asigna** | ICMP 18608*          | Persia americana       | JX010244                |
| **C. arecolinum**        | ICMP 17673* ATCC 201874 | Arecolinum virginicum | JX010176                |
| **C. alatae**            | CBS 304.67* ICMP 17919 | Dinoseira alata        | JX010190                |
| **C. alienum**           | ICMP 12071*          | Malus domestica        | JX010251                |
| **C. aotearoa**          | ICMP 18735           | Hedychium gardnerianum | JX010221                |
| **C. arecicola**         | hb8                  | Areca catechu          | MW557482                |
| **C. aesculina**         | ICMP 1861167*        | Aesculus hirsuteoideus | MN415991                |
| **C. asiaticum**         | ICMP 15808* CBS 130418 | Coffea arabica        | JX010265                |
| **C. australum**         | BRIP 16695           | Capisicum annuum       | KU923677                |
| **C. boninense**         | CBS 123755*          | Grindina asiaticum var. sinicum | JQ005153                |
| **C. camelliae**         | ICMP 10643           | Camellia × williamsii  | JX010224                |
| **C. chonggongensis**    | MFLUCC 15-0022*      | Prangrea × ananassia   | KP83152                 |
| **C. chongnsisense**     | MFLUCC 18-0945       | Magnolia grandiflora   | MW346499                |
| **C. chrysophilum**      | MCM 4268*            | Musa sp.               | KO094252                |
| **C. coccifera**         | ICMP 19212           | Vaccumium sp.          | JX010228                |
| **C. clidemiae**         | ICMP 18658*          | Clidemia hirta         | JX010265                |
| **C. coclibonense**      | BRIP 166219          | Condylina stricta × Condylina australis | MH080716                |
| **C. cosmetics**          | CAUG17*              | Capisicum annuum       | KP890168                |
| **C. conyliniola**       | MFLUCC0955* ICMP 18579 | Conyliniola fruticosa | JX010226                |
| **C. dncan.geniculum**   | MFLUCC 19-0430*      | Dracaena fragrans     | MN921255                |
| **C. endolphiota**       | CAUG8                | Capisicum annuum       | KP145441                |
| **C. fci-septicae**      | MFLU 19-27708*       | Ficus septica          | MW114367                |
| **C. fructicola**        | MFLU 09022*          | Coffea arabica         | FJ792603                |
| **C. fructivorum**       | CBS 13-3125*         | Vaccinium macrocarpon | JX141545                |
| **C. gloeosporoidei**    | IMI156878* ICMP 17821 | Citrus sinensis      | JX01052                |
| **C. gloeosporioides**   | ICMP 19212           | Citrus limon           | JX01018                 |
| **SAUCCL 200952**        | Juglans regia        | Juglans regia          | MW786743                |
| **SAUCCL 200954**        | Juglans regia        | Juglans regia          | MW786745                |
| **SAUCCL 201001**        | Juglans regia        | Juglans regia          | MW786747                |
| **C. grevilleae**        | CBS 132879*          | Grevillea sp.          | KC297078                |
| **C. granicum**          | CAUG7*               | Capisicum sp.          | KP890165                |
| **C. hebriseae**         | MFLUCC 30-726*       | Vaius vivifera         | FK15683                 |
| **C. heidelbergia**      | MFLU 15-0689         | Hedera helix           | MG33184                 |
| **C. helenii**           | CBS 142148*          | Poncirus trifliflata   | KY856446                |
| **C. hematoxylon**       | LF238*               | Camellia sinensis      | KJ955109                |
| Species          | Strain/Isolate | Host/Substrate | GenBank accession number                  |
|------------------|----------------|----------------|------------------------------------------|
|                  |                |                | ITS  | GAPDH  | CH3-1 | ACT  | TUB2 | CAL  | GS  |
| C. horii         | ICMP 10492    | Diospyros kaki | GQ329690 | GQ329681 | JX009197 | JX00938 | JX010450 | JX010640 | JX010137 |
| C. hybricus      | CPC 28153*    | Citrus hybridis| KY856450 | KY856274 | KY856190 | KY856023 | KY856552 | -        | -    |
| C. japonica      | LF687*        | Camellia sinensis| KJ955201 | KJ954902 | KJ954747 | KJ955348 | KJ954752 | KJ955051 |
| C. kahawae       | IMI 319418*   | Coffea arabica | JX010231 | JX010012 | JX009813 | JX009452 | JX010444 | -        | JX010130 |
| C. ledongense    | CGMCC3.18888*| Quercus palustris| MG242080 | MG242016 | MG242018 | MG242014 | MG242010 | -        | -    |
| C. malasseae     | CBS 143664a*  | Capricium annuum| MH28812 | MH288220 | MH805850 | MH781480 | MH846563 | -        | -    |
| C. menginense    | SAUCC200702*  | Rosa chinensis | MW876474 | MW8646240 | MW883686 | MW883695 | MW8885970 | MW922538 | MW888961 |
|                  | SAUCC200912   | Juglans regia | MW876649 | MW876472 | MW883687 | MW883696 | MW8885972 | MW922539 | MW888962 |
|                  | SAUCC200913   | Juglans regia | MW876690 | MW876473 | MW883688 | MW883697 | MW8885972 | MW922540 | MW888963 |
|                  | SAUCC200983   | Juglans regia | MW876682 | MW876475 | MW883691 | MW883699 | MW8885975 | MW922543 | MW888966 |
| C. musae         | CBS 116870*   | Musa sp.       | JX010146 | JX010050 | JX009896 | JX009433 | -        | -        | -    |
| C. nupharicola   | CBS 17096*    | Nuphar lutea subsp. polypetala | JX010187 | JX009972 | JX009835 | JX009437 | JX010398 | JX010663 | JX010088 |
| C. pandanisola   | MFLU 18-0003*| Pandanus sp.   | MG646967 | MG646934 | MG646931 | MG646926 | -        | -        | -    |
|                  | SAUCC200204   | Juglans regia | MW876646 | MW876478 | MW883693 | MW883702 | MW8885977 | MW922545 | MW888960 |
| C. persea        | GA100*        | Persea americana | KX620308 | KX620242 | KX620145 | KX620341 | KX620206 | KX620275 |
| C. pontosus      | CBS 132882*   | Pinnaceae sp.  | KC297079 | KC297009 | KC296986 | KC296940 | KC297101 | KC296960 | -    |
| C. pseudobromeliana | MFLUCC 18-1602 | Prunus avium | MH813785 | MH853675 | MH853678 | MH853681 | -        | -        | -    |
| C. pseudobromeliana | MFLUCC 18-1602 | Prunus avium | MH813785 | MH853675 | MH853678 | MH853681 | -        | -        | -    |
| C. pseudobromeliana | MFLUCC 18-1602 | Prunus avium | MH813785 | MH853675 | MH853678 | MH853681 | -        | -        | -    |
| C. pseudobromeliana | MFLUCC 18-1602 | Prunus avium | MH813785 | MH853675 | MH853678 | MH853681 | -        | -        | -    |
| C. pseudobromeliana | MFLUCC 18-1602 | Prunus avium | MH813785 | MH853675 | MH853678 | MH853681 | -        | -        | -    |
| C. queenslandicum | ICMP 17878*   | Carica papaya  | JX00238 | JX009987 | JX009349 | JX00947 | JX010443 | JX010037 | -    |
| C. rhoeae        | CBS 133134*   | Rheca virgincia| JX145128 | JX145128 | JX145128 | JX145128 | -        | -        | -    |
| C. salolae       | ICMP 1005*    | Salola tragaia | JX010424 | JX009916 | JX009863 | JX010430 | -        | -        | -    |
| C. siamense      | ICMP 18578*   | Coffea arabica | JX10071 | JX009924 | JX009865 | JX010430 | JX970423 | JX970424 | JX970425 |
| C. theobromicola | ICMP 19118    | Jasminum sambac| HM133151 | HM133149 | HM805850 | HM133107 | HM104151 | JX100105 | -    |
| C. theobromicola | ICMP 19118    | Jasminum sambac| HM133151 | HM133149 | HM805850 | HM133107 | HM104151 | JX100105 | -    |
| C. tropica       | CBS 124949*   | Theobroma cacao| JX010294 | JX010006 | JX009869 | JX009444 | JX100447 | JX009590 | JX010139 |
| C. uvarum        | ICMP 18649    | Theobroma cacao| JX010264 | JX010002 | JX009807 | JX009489 | JX100407 | JX009719 | JX010097 |
| C. xanthorrhoeae  | BRIP 45094*   | Xanthorrhoea preissii| JX010261 | JX009927 | JX009823 | JX009478 | JX100448 | JX009653 | JX010138 |
| C. yulongense    | CFCC 30818*   | Vaccinium dunalianum| MH751307 | MK108986 | MH793605 | MH773394 | MK108987 | MH793604 | MK108988 |
| Calletotrichum sp. | BRIP 58074a | Citrus aurantifolia | MK469999 | MK470017 | MK470035 | MK470053 | -        | -        | -    |

Strains marked with "*" are ex-type or ex-epitype.
Results

Phylogenetic analyses

Nine strains of *Colletotrichum* isolated from leaves of *Rosa chinensis* and fruit of *Juglans regia* in Mengyin County, Shandong Province, China, were grown in culture. Among the nine *Colletotrichum* isolates were identified a new species and two known species based on an analysis of combined ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS gene sequences composed of 69 isolates of *C. gloeosporioides* species complex and *C. boninense* (CBS 123755) as the outgroup taxon.

A total of 3953 characters including gaps were obtained in the phylogenetic analysis, viz. ITS: 1–619, GAPDH: 620–929, CHS-1: 930–1229, ACT: 1230–1542, TUB2: 1543–2288, CAL: 2289–3028, GS: 3029–3953. Of these characters, 2667 were constant, 674 were variable and parsimony-uninformative, and 612 were parsimony-informative.

The Bayesian analysis lasted 4,685,000 generations, resulting in 4686 total trees, of which 3515 trees were used to calculate the posterior probabilities. The BI posterior probabilities were plotted on the ML tree. For the BI and ML analyses, HKY+G for GAPDH and ACT, SYM+I+G for ITS, K80+I+G for CHS-1, GTR+G for GS and CAL, HKY+I for TUB2 were selected and incorporated into the analyses. The ML tree topology confirmed the tree topologies obtained from the BI analyses, and therefore, the ML tree is presented (Fig. 1).

ML bootstrap support values (≥ 50%) and Bayesian posterior probability (≥ 0.90) are shown as first and second position above nodes, respectively. The 70 strains were assigned to 60 species clades based on the seven gene loci phylogeny (Fig. 1). The nine strains studied here represented a novel species and two known species. The new species of *C. mengyinense* showed a close relationship to *C. fructicola* (MFLU 090228) with full support (ML-BS: 100% and BYPP: 1). The strains SAUCC200952, SAUCC200954 and SAUCC201001 belong to *C. gloeosporioides* (IMI356878) with full support (ML-BS: 100% and BYPP: 1) by the multi-locus phylogeny. The strains SAUCC200204 and SAUCC201152 belong to *C. pandanicola* (MFLU 18-0003) with good support (ML-BS: 94% and BYPP: 0.99) by the multi-locus phylogeny.

Taxonomy

*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., Atti Reale Ist. Veneto Sci. Lett. Arti., ser. 6, 2: 670. 1884

Figure 2

*Vermicudaria gloeosporioides* Penz., Michelia 2: 450, 1882. Basionym.

Description. Lesion fruit, round or irregular, dark brown slightly sunken center, brown at margin. Asexual morph developed on PDA. A mass of orange conidia grows in the white my-
Figure 1. Phylogram of Colletotrichum gloeosporioides complex based on combined ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS genes. The ML and BI bootstrap support values above 50% and 0.90 BYPP are shown at the first and second position, respectively. Strains marked with “*” are ex-type or ex-epitype. Strains from this study are shown in red. Two branches were shortened to fit the page size—these are indicated by the symbol (//) with an indication number showing how many times they are shortened.
Colletotrichum in Shandong province, China

Celium of PDA after 14 days in light at 25 °C. Conidia, hyaline, smooth-walled, subcylindrical, both ends round, 1–3-guttulate, contents granular. Conidia on PDA (10.6–16.5 × 4.3–5.3 μm, mean ± SD = 14.9 ± 1.5 × 4.9 ± 0.3 μm, L/W ratio = 3.0, n = 40). Sexual morph not observed. Conidiogenous cells subcylindrical, straight to curved, 4.7–12.7 × 3.1–4.0 μm, opening 1.5–2.0 μm diam. Conidiophores hyaline, smooth walled, septate, branched.

Culture characteristics. Colonies on PDA flat with entire margin, aerial mycelium white, floccose cottony; surface and reverse grayish in the center and white margin. PDA attaining max 81 mm in diameter after 7 days, at 25 °C, growth rate 8.7–11.5 mm/day. Colonies on SNA sparse hyphae, slow growth.

Specimens examined. China, Shandong Province: Mengyin County, Mengshan, on diseased fruit of Juglans regia, 25 July 2020, T.C. Mu, paratype HSAUP200952, ex-paratype living culture SAUCC200952. China, Shandong Province: Mengyin

Figure 2. Colletotrichum gloeosporioides (SAUCC201001) a lesion fruit of host plant b, c surface (b) and reverse (c) sides of colony after incubation for 7 days on PDA d conidiomata e conidiophores, conidiogenous cells and conidia f–h conidia. Scale bars: 10 μm (e–h).
Notes. *Colletotrichum gloeosporioides* was originally described as *Vermicularia gloeosporioides* on fruit of *Citrus sinensis* in Italy and this species placed in *Colletotrichum* by Corda (Weir et al. 2012; Cannon et al. 2008). In the present study, three strains (SAUCC200952, SAUCC200954 and SAUCC201001) are clustered to *C. gloeosporioides* clade in the combined phylogenetic tree (Fig. 1). Morphologically, our strains were similar to *C. gloeosporioides* by conidia (10.6–16.5 × 4.3–5.3 μm vs. 12.0–17.0 (–23.5) × 4.5–6.0 μm, mean: 14.9 × 4.9 vs. 14.4 × 5.6 μm). We therefore consider the isolated strain as *C. gloeosporioides*.

*Colletotrichum mengyinense* T.C. Mu, J.W. Xia, X.G. Zhang & Z. Li, sp. nov.

MycoBank No: 841265

Figure 3

**Etymology.** Named after Mengyin County where the fungus was collected.

**Diagnosis.** *Colletotrichum mengyinense* can be distinguished from the phylogenetically most closely related species *C. fructicola* (MFLU 090228) by its large conidia (12.5–15.7 × 4.8–6.1 vs. 9.7–14.0 × 3.0–4.3 μm), and five loci (2/509 in the ITS region, 1/139 GAPDH, 9/237 ACT, 8/410 TUB2 and 20/727 GS).

**Type.** China, Shandong Province: Mengyin County, on diseased leaves of *Rosa chinensis*, 25 July 2020, T.C. Mu, holotype HSAUP200702, ex-type living culture SAUCC200702.

**Description.** Leaf spots discoid to irregular, brown or tanned. Asexual morph developed on SNA. A yellowish or orange mass appearing just as accumulations of conidia on the surface of the medium of SNA after 14 days in light at 25 °C. Conidia one-celled, hyaline, smooth-walled, subcylindrical, both ends round, contents granular. Conidia on SNA (12.5–15.7 × 4.8–6.1 μm, mean ± SD = 14.3 ± 1.1 × 5.3 ± 0.4 μm, L/W ratio = 2.7, n = 40). Sexual morph not observed. Conidiogenous cells subcylindrical, hyaline, 5.3–15.5 × 2.9–4.9 μm, opening 1.7–2.5 μm diam. Conidiophores hyaline, smooth walled, septate, branched.

**Culture characteristics.** Colonies on PDA flat with entire margin, aerial mycelium white or gray, floccose cottony; surface and reverse gray in the center and grayish margin. PDA attaining 69.3–75.6 mm in diameter after 7 days, at 25 °C, growth rate 9.9–10.8 mm/day. Colonies on SNA sparse hyphae, slow growth.

**Additional specimen examined.** China, Shandong Province: Mengyin County, on diseased fruit of *Juglans regia*, 25 July 2020, T.C. Mu, paratype HSAUP200912, ex-paratype living culture SAUCC200912. China, Shandong Province: Mengyin County, on diseased fruit of *Juglans regia*, 25 July 2020, T.C. Mu, paratype HSAUP200913, ex-paratype living culture SAUCC200913. China, Shandong Province: Mengyin County, on diseased fruit of *Juglans regia*, 25 July 2020, T.C. Mu, paratype HSAUP200983, ex-paratype living culture SAUCC200983.
Phylogenetic analysis of a combined seven gene showed that *Colletotrichum mengyinense* formed an independent clade (Fig. 1) and is phylogenetically distinct from *C. fructicola* (Prihastuti et al. 2009). This species can be distinguished from *C. fructicola* by 40 different nucleotides (2/509 in the ITS region, 1/139 in the GAPDH region, 9/237 ACT, 8/410 TUB2 and 20/727 GS). What's more, *C. mengyinense* differs from *C. fructicola* in having large conidia (12.5–15.7 × 4.8–6.1 vs. 9.7–14.0 × 3.0–4.3 μm, mean: 14.3 × 5.3 vs. 11.53× 3.55 μm). Therefore, we establish this fungus as a novel species.

*Colletotrichum pandanicola* Tibpromma & K.D. Hyde, MycoKeys 33:47. (2018)  
Figure 4

Description. Lesion fruit, round or irregular, dark brown slightly sunken center, brown at margin. Asexual morph developed on SNA. A mass of yellowish or orange

**Figure 3.** *Colletotrichum mengyinense* (SAUCC200702) a branch with leaves of host plant b, c surface (b) and reverse (c) sides of colony after incubation for 7 days on PDA d conidiomata e-g conidiophores, conidiogenous cells and conidia h–j conidia. Scale bars: 10 μm (e–j).
creamy conidial droplets at the inoculum point on SNA after 14 days in light at 25 °C. Born in conidiomata, conidia first take an ovoid shape, then become subcylindrical with rounded ends, contents granular. Conidia on SNA (14.2–17.9 × 4.6–6.0 μm, mean ± SD = 16.1 ± 0.9 × 5.4 ± 0.3 μm, L/W ratio = 2.9, n = 40). Sexual morph not observed. Conidiogenous cells subcylindrical, hyaline, 5.5–23.9 × 2.6–6.3 μm, opening 1.1–1.5 μm diam. Conidiophores branched, hyaline, smooth walled, septate, some septa disappeared at the end, contents granular.

**Culture characteristics.** Colonies on PDA flat with entire margin, aerial mycelium white, floccose cottony; light gray in the center and pale white margin, reverse white to pale brownish. PDA attaining 58.1–82.6 mm in diameter after 7 days, at 25 °C, growth rate 8.3–11.8 mm/day. Colonies on SNA sparse hyphae, slow growth.
Specimens examined. China, Shandong Province: Mengyin County, Mengshan, on diseased fruit of *Juglans regia*. 25 July 2020, T.C. Mu, paratype HSAUP200204, ex-paratype living culture SAUCC200204. China, Shandong Province: Mengyin County, Mengshan, on diseased fruit of *Juglans regia*. 25 July 2020, T.C. Mu, paratype HSAUP201152, ex-paratype living culture SAUCC201152.

Notes. *Colletotrichum pandanicola* was originally described from the healthy leaves of *Pandanus* sp. (MFLU 18-0003, Pandanaceae) in Thailand (Tibpromma et al. 2018). In the present study, two strains (SAUCC200204 and SAUCC201152) are clustered to the *C. pandanicola* clade in the combined phylogenetic tree (Fig. 1). Morphologically, our strains were similar to *C. pandanicola* by conidia (14.2–17.9 × 4.6–6.0 vs. 9.0–18.0 × 4.0–8.0 μm, mean: 16.1 × 5.4 vs. 13.39 × 5.35 μm). We therefore consider the isolated strains as *C. pandanicola*.

Discussion

In this study, the *Colletotrichum* specimens of diseased leaves and fruits were collected in Mengyin, Shandong Province, China. A temperate monsoon climate and an abundance of fruit trees provide the proper conditions for anthracnose propagation. As a result, 70 reference sequences (including an outgroup taxon: *C. boninense* CBS 123755) were selected based on BLAST searches of NCBI’s GenBank nucleotide database and were included in the phylogenetic analyses (Table 2).

Phylogenetic analyses based on seven combined loci (ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS), as well as morphological characters of the asexual morph obtained in culture, were contributed to knowledge of the diversity of *Colletotrichum* species in Shandong Province. Based on a large set of freshly collected specimens from Shandong province, China, nine strains of *Colletotrichum* species were isolated from two host genera (Table 2). A new species is proposed: *C. mengyinense*. In a previous report, *C. gloeosporioides* has been isolated from *Juglans regia* (Zhu et al. 2014). *Colletotrichum pandanicola* was described from *Pandanus* sp. (Pandanaceae) in Thailand (Tibpromma et al. 2018) and *C. pandanicola* is first reported from *Juglans regia* in China. In this study, we described and illustrated *C. gloeosporioides* and *C. pandanicola* again.

Previously, species identification of *Colletotrichum* was largely referred to the host-specificity and pure culture characteristics, leading to the chaos of names (Weir et al. 2012). On the other hand, based on a polyphasic approach and known morphology, more than one species of *Colletotrichum* can colonize a single host, while one species can be associated with different hosts (Damm et al. 2012). It revealed diversity of *Colletotrichum* species from different hosts. Our study supported this result. For example, *C. pandanicola* (SAUCC200204 and SAUCC201152) and *C. gloeosporioides* (SAUCC200952, SAUCC200954 and SAUCC201001) were collected from *Juglans regia*. In addition, isolates of *C. mengyinense* were obtained from two hosts (*Juglans regia* and *Rosa chinensis*). The morphological descriptions and molecular data for species of *Colletotrichum* represent an important resource and basis for plant pathologists and fungus taxonomists.
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