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Characterization of the causal agent of poplar anthracnose occurring in the Beijing region

Zheng Li[1], Ying Mei Liang[2] & Cheng Ming Tian[1*]

[1]*Key Laboratory for Silviculture and Conservation of Ministry of Education, Beijing Forestry University &* [2]*Museum of Beijing Forestry University, Beijing 100083, P. R. China*

* Correspondence to: *chengmt@bjfu.edu.cn*

Abstract — Twenty fungal isolates derived from infected poplar leaves collected in four Beijing regional districts during 2009 and 2010 were examined for morphological and cultural characteristics. Multi-gene phylogenetic sequence analyses of the rDNA ITS (ITS1-5.8S-ITS2) region and the β-tubulin2, partial actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GPDH), and glutamine synthetase (GS) genes were performed for these isolates. The morphological and cultural evaluations and DNA sequence analyses demonstrated that 16 isolates represented *Colletotrichum gloeosporioides* while the remaining four isolates, all from Shi Jingshan district, represent a new species, *Colletotrichum populi*, which is described and illustrated.

Key words — disease, multi-locus phylogeny, taxonomy

Introduction

The genus *Colletotrichum* causes anthracnose disease on a wide range of plants worldwide. The main *Colletotrichum* species causing anthracnose of forest trees include *C. gloeosporioides*, *C. acutatum,* and *C. crassipes*, which may cause infections of leaves and fruits of plants such as poplar, China fir, and paulownia, often leading to premature defoliation (Xu et al. 2004). Of these, *C. gloeosporioides* is the most common, affecting a wide range of tree hosts. The taxonomy of *C. gloeosporioides* is extremely complex because of its heterogeneous nature and instability in cultural characteristics. For example, more than 600 *C. gloeosporioides* synonyms have been identified (von Arx 1957). Different experimental conditions and measurement errors may have a marked influence over the recorded sizes of conidia. Identification results obtained through traditional morphological examination are thought to be unreliable (Cai et al. 2009) when compared to molecular methods, and several new species, such as *C. asianum* and *C. cordylinicola* (Prihastuti et al. 2009,

Table 1. Isolates of *Colletotrichum* used in study.

Isolate No.	Host	Origin	Sampling date
1C5-7	*Populus ×beijingensis*	Chang ping	2010.08
C1-5-1	*Populus ×beijingensis*	Chang ping	2010.08
2C5-8	*Populus ×beijingensis*	Chang ping	2010.08
2C5-9	*Populus ×beijingensis*	Chang ping	2010.08
2C5-14	*Populus ×beijingensis*	Chang ping	2010.08
3C5-3	*Populus ×beijingensis*	Chang ping	2010.08
4C5-2	*Populus ×beijingensis*	Chang ping	2010.08
C2-5	*Populus ×beijingensis*	Chang ping	2010.07
Y3-4	*Populus cathayana*	Yan qing	2010.08
Y3-6	*Populus cathayana*	Yan qing	2010.08
Yp1-7	*Populus cathayana*	Yan qing	2010.08
Bh7-2	*Populus ×beijingensis*	Yan qing	2010.09
Bh9-1	*Populus cathayana*	Yan qing	2010.09
Bh12-2	*Populus ×beijingensis*	Yan qing	2010.09
Dy10-2	*Populus nigra*	Yan qing	2010.09
HMBFU191	*Populus nigra* var. *italica*	Shi Jingshan	2009.10
HMBFU163	*Populus nigra* var. *italica*	Shi Jingshan	2009.10
HMBFU173	*Populus nigra* var. *italica*	Shi Jingshan	2009.10
HMBFU141	*Populus nigra* var. *italica*	Shi Jingshan	2009.10
M1-6	*Populus nigra*	Mi yun	2010.08

Phoulivong et al, 2010a), have recently been separated from *C. gloeosporioides* based on the results from molecular analysis.

Colletotrichum gloeosporioides (teleomorph *Glomerella cingulata*) has been reported as the main pathogen of poplar anthracnose in China and North America (He et al. 1990, Newcombe 2000). *Colletotrichum graminicola*, which usually infects monocotyledonous plants such as cereals or grasses, may also infect poplar in India (Dipak et al. 1999). Most studies that are based on morphology may be contradicted by molecular data (Phoulivong et al. 2010b).

The present study was carried out to determine the *Colletotrichum* species associated with poplar anthracnose in Beijing area through morphological examination and molecular methods.

Materials & methods

Isolation of *Colletotrichum* species

Twenty *Colletotrichum* isolates were obtained from lesions on infected leaves of different poplar species collected in four districts in Beijing: Chang ping, Yan qing, Mi yun, and Shi Jingshan (Table 1). These isolates are deposited at the Mycological Herbarium of Beijing Forestry University (BJFC).

Three 5×5 mm samples cut from the interface of healthy and diseased tissue were surface sterilized by dipping in 70% ethanol for 1 minute, then in 1% sodium hypochlorite for 3–4 minutes and washing in three changes of sterile water. The leaf segments were then placed onto the surface of potato dextrose agar (PDA) and incubated

at room temperature (28°C). The growing edges of fungal hyphae developing from the leaf segments were transferred onto a new PDA plate after 1–2 days. Each isolate was purified through single spore isolation and was maintained on a new PDA plate at 28°C for further study.

Morphological studies

Mycelial discs (5 mm diam) derived from the edge of a 7-day old culture were transferred onto PDA and incubated at 28°C in the dark. Each colony radius was measured daily after the 2nd day for 4 days to determine the growth rate by averaging the daily growth (cm per day) of the colonies. Colony characters were also recorded and described. After 7 days a spore suspension (5×10^5 conidia/ml) was made from each culture and the size and shape of 30 conidia were measured by light microscopy at a magnification of 40x. A drop of the spore suspension was placed on a slide, which was placed in a Petri dish containing water-soaked filter paper to keep moist. After 24 hours (at 28°C) conidial germination was observed, and the size and shape of 20 appressoria were measured.

Statistical data (morphology and growth rate) were analyzed using SPSS software version 11.5.0 (SPSS Inc., USA).

DNA extraction and PCR amplification

Mycelium was scraped from the surface of 7-day old cultures of each isolate and dried using sterilized filter paper. Genomic DNA was extracted using the modified CTAB method. Extracted DNA was electrophoresed on 1% agarose gel to check genomic DNA quality.

Using polymerase chain reaction (PCR), DNA was amplified from the complete rDNA-ITS (ITS) region and the β-tubulin (TUB2), partial actin (ACT), glutamine synthetase (GS), and glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes of each *Colletotrichum* isolate. The primer pairs for the amplified genes are ITS1+ITS4 (White et al. 1990), Bt2a+Bt2b (Glass et al. 1995), ACTF+ACTR (Carbone & Kohn 1999), GSF1+GSR1 (Guerber et al. 2003), GDF1+GDR1 (Peres et al. 2008) respectively. Amplification was performed in 25 μL volume with 1 μL (20 ng) DNA template, 2 μL dNTP (2.5 mmol/L), 2.5 μL 10×PCR buffer, 0.5 μL of each primer (10 μLmol/L), 0.15 μL Taq DNA polymerase and 18.35 μL ddH_2O. The PCR profile consisted of denaturation at 95°C for 3 min, followed by 34 cycles at 95°C for 1 min, 55°C for 30 s, 72°C for 1 min and a final cycle at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gel and DNA sequencing was carried out by the Shanghai Invitrogen Biological Technology Co. Errors were adjusted manually with BioEdit software version 7.0.5.3 (© Tom Hall). All isolate and DNA sequence accession (DDBJ, EMBL, GenBank) numbers are listed in Table 2.

Phylogenetic analysis

The sequences of *Colletotrichum* isolates were aligned using Clustal X (1.81). Alignments were manually adjusted to allow maximum alignment and maximum sequence similarity, and gaps were treated as missing data.

For parsimony analysis, PAUP version 4.0b10 was used and heuristic search was performed with 1000 repeats of random addition sequences in Stepwise-Addition Option and TBR swapping algorithm in Branch Swapping Option. Confidence limits

Table 2. *Colletotrichum* isolates used in this study.*

Species	Isolate No.	Host	Location	Database accession No. ^				
				ITS	β-tubulin	ACT	GPDH	GS
C. asianum	BPD-I4	Coffee	Thailand	FJ972612	FJ907439	FJ907424	FJ972576	FJ972595
C. acutatum	CBS294.67	Papaya	Australia	FJ972610	FJ907444	FJ907429	FJ972581	FJ972590
C. boninense	C05016	Ginseng	Korea	GU935883	GU935903	GU935804	GU935863	GU935823
C. cordylinicola	LC0856	*Cordyline fruticosa*	Thailand	HM470246	HM470249	HM470234	HM470240	HM470243
C. fructicola	BPD-I12	Coffee	Thailand	FJ972611	FJ907440	FJ907425	FJ972577	FJ972594
C. gloeosporioides	CBS953.97	Citrus	Italy	FJ972609	FJ907445	FJ907430	FJ972582	FJ972589
C. gloeosporioides	**Bh9-1**	**Poplar**	**China**	**AB632351**	**JN862897**	**JN184702**	**JN211078**	**JN211087**
C. gloeosporioides	**4C5-2**	**Poplar**	**China**	**AB632352**	**JN862882**	**JN184700**	**JN211065**	**JN211097**
C. gloeosporioides	**Bh7-2**	**Poplar**	**China**	**AB632353**	**JN862883**	**JN184701**	**JN211080**	**JN211091**
C. gloeosporioides	**Yp1-7**	**Poplar**	**China**	**AB632354**	**JN862884**	**JN184698**	**JN211075**	**JN211095**
C. gloeosporioides	**1C5-7**	**Poplar**	**China**	**AB632355**	**JN862885**	**JN184699**	**JN211074**	**JN211086**
C. gloeosporioides	**2C5-9**	**Poplar**	**China**	**AB632356**	**JN862886**	**JN184697**	**JN211073**	**JN211090**
C. gloeosporioides	**Dy10-2**	**Poplar**	**China**	**AB632357**	**JN862896**	**JN184692**	**JN211077**	**JN211092**
C. gloeosporioides	**3C5-3**	**Poplar**	**China**	**AB632358**	**JN862887**	**JN184705**	**JN211072**	**JN211098**
C. gloeosporioides	**Y3-6**	**Poplar**	**China**	**AB632359**	**JN862895**	**JN184694**	**JN211071**	**JN211093**
C. gloeosporioides	**C2-5**	**Poplar**	**China**	**AB632360**	**JN862894**	**JN184695**	**JN211070**	**JN211088**
C. gloeosporioides	**2C5-14**	**Poplar**	**China**	**AB632361**	**JN862893**	**JN184696**	**JN211076**	**JN211099**
C. gloeosporioides	**Y3-4**	**Poplar**	**China**	**AB632362**	**JN862892**	**JN184689**	**JN211069**	**JN211085**
C. gloeosporioides	**M1-6**	**Poplar**	**China**	**AB632363**	**JN862891**	**JN184690**	**JN211066**	**JN211094**
C. gloeosporioides	**2C5-8**	**Poplar**	**China**	**AB632364**	**JN862890**	**JN184691**	**JN211068**	**JN211096**
C. gloeosporioides	**BH12-2**	**Poplar**	**China**	**AB632365**	**JN862889**	**JN184687**	**JN211079**	**JN211100**
C. gloeosporioides	**C1-5-1**	**Poplar**	**China**	**AB632366**	**JN862888**	**JN184688**	**JN211067**	**JN211089**
C. horii	TSG002	*Mangifera indica*	China	AY791890	GU13380	GU13337	GQ329680	GU133381
C. hymenocallidis	CSSN3	*Hymenocallis*	China	GQ485601	GQ849439	GQ856776	GQ856759	—
C. jasmini-sambac	LLTX-01	*Jasminum sambac*	China	HM131511	HM153768	HM131507	HM131497	HM131502
C. kahawae	IMI319418	Coffee	Kenya	FJ972608	FJ907446	FJ907432	FJ972583	FJ972588
C. populi	**HMBFU191**	**Poplar**	**China**	**AB632347**	**JN862898**	**JN184704**	**JN211081**	**JN211101**
C. populi	**HMBFU163**	**Poplar**	**China**	**AB632348**	**JN862899**	**JN184706**	**JN211082**	**JN211102**
C. populi	**HMBFU173**	**Poplar**	**China**	**AB632349**	**JN862900**	**JN184707**	**JN211084**	**JN211104**
C. populi	**HMBFU141**	**Poplar**	**China**	**AB632350**	**JN862901**	**JN184708**	**JN211083**	**JN211103**
C. siamense	BML-115	Coffee	Thailand	FJ972614	FJ907437	FJ907422	FJ972574	FJ972597

* Isolates and sequences generated by this research are indicated in bold. ^ Accession numbers are all available in DDBJ (DNA Database of Japan), EMBL, and GenBank.

for the branches based on parsimony criteria were estimated by bootstrap analysis of 1000 replicates. Phylogenetic trees were constructed from distance matrix values by the maximum parsimony (MP) method. Describing trees was based on tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI). Constructed trees were viewed by TreeView software. The GenBank accession numbers used as references in this study are listed in Table 2.

Results

Morphological and cultural characterization

The isolates could be divided into two groups (Table 3) based on differences in cultural characteristics and appressorial morphology. Group 1 consisted of 16 isolates with colonies varying from white or grey to dark brown and turning dark grey or olive-green with time; aerial mycelium white or grey (Plate 1a–b). These cultures produced abundant orange conidial masses around the inoculation point. The conidia were cylindrical, obtuse at both ends or slightly tapered at one end, possessing 2–3 vacuoles and measuring 9.8–22.0 × 2.9–6.9 μm (Plate 1c). Appressoria were generally round or ovate and 4.9–15.9 × 4.4–9.8 μm (Plate 1d).

Table 3. Morphological and cultural characteristics of 20 *Colletotrichum* isolates. *

	C. gloeosporioides (Group 1)	*C. populi* (Group 2)
Isolates Tested	16	4
Conidia		
Shape	Cylindrical	Cylindrical
Length (μm)	15.8 ± 1.9 (9.8–22.0)	15.1 ± 2.0 (9.8–17.7)
Width (μm)	4.9 ± 0.4 (2.9–6.9)	5.2 ± 0.9 (3.0–7.3)
Appressoria		
Shape	Circular or ovate	Irregular, edge lobed
Length (μm)	9.4 ± 2.1 (4.9–15.9)	9.4 ± 1.6 (7.3–12.2)
Width (μm)	5.9 ± 1.0 (4.4–9.8)	6.2 ± 1.2 (4.9–9.3)
Colony	Light, dark grey or brown; aerial mycelium grey with orange conidial masses	Aerial mycelium cottony, white, with indistinct conidial masses
Daily Growth (cm/d)	0.72 ± 0.08 (0.56–0.88)	0.73 ± 0.05 (0.62–0.80)

* Measurements are presented as mean ± sd (range).

Group 2 consisted of four isolates collected from Shi Jingshan district. The colonies were white to light grey in color and covered with white cottony aerial mycelia, with indistinct conidia masses on the surface of colonies. Conidia were similar to those of Group 1 in shape (cylindrical) and size (9.8–17.7 × 3.0–7.3 μm), but had 1–2 vacuoles. Appressoria were irregular in shape with lobed edges and measured 7.3–12.2 × 4.9–9.3 μm.

There were no significant differences in growth rate between the two groups, both showing an average daily growth of ca. 0.72 cm/d.

Multi-locus phylogeny

The sequence information obtained from five genes and the phylogenetic tree information based on each gene sequence are shown in Table 4. Of the five genes, GS and GPDH had the highest rates of parsimony informative characters (PIC), GS comprising 439 (40.1%) PIC out of a total 1095 characters and GPDH 114 (38.6%) PIC out of 295 characters.

The phylogenetic tree was constructed on the basis of combined data of the five genes (Plate 2). The best tree contained 2593 characters with NPIC=681, CI=0.865, RI=0.853, HI=0.135, RC=0.738 and Length=1712, showing that the two morphologically distinct groups were placed into two separate branches. Group 1 clustered with *C. gloeosporioides* with a bootstrap value of 100%. Group 2 was distinctive from *C. fructicola* (bootstrap value = 79%) and other members in '*gloeosporioides* complex' (bootstrap values > 90%), suggesting the probability of a new species.

Table 4. Gene regions amplified.

DNA region	Sequence lengths (BP)	Phylogenetic tree		
		Characters	NPIC	NPIC/Characters (%)
ITS	534–537	563	45	8.0
TUB2	440–441	457	84	18.4
ACT	241	299	60	20.1
GPDH	221–230	295	114	38.6
GS	981–988	1095	439	40.1

Taxonomy

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. Plate 1

Lesions on leaves of poplar as black spots distributed randomly or in circles with a dark brown border, orange conidial masses sometimes observed. Conidia cylindrical, 9.8–22.0 × 2.9–6.9 μm, obtuse at ends, hyaline, smooth. Appressoria ovate or circular, 4.9–15.9 × 4.4–9.8 μm. Colonies on PDA with white, grey, dark grey, or olive grey mycelium and orange sporulation. Reverse brown or olive-green with dark spots.

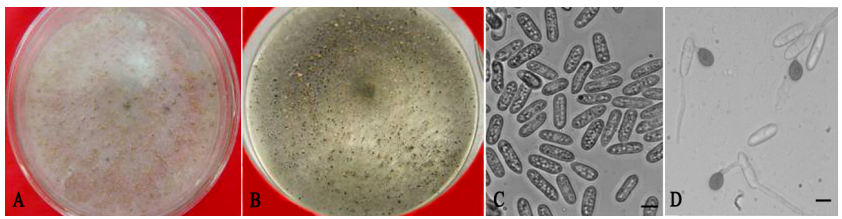

Plate 1. *Colletotrichum gloeosporioides* isolates (Group 1): cultural and morphological characters. A: Colony of isolate 2C5-14; B: Colony of isolate BH7-2; C: Conidia of isolate M1-6; D: Clavate or circular appressoria of isolate C1-5-1. Bars = 10 μm.

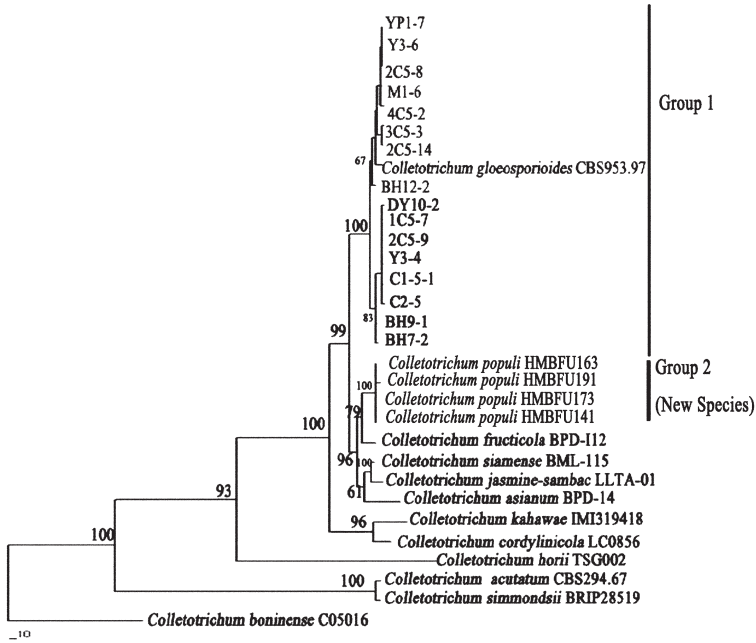

Plate 2. Phylogenetic tree based on the combination of sequences of ITS, TUB2, ACT, GPDH, and GS genes (MP.)

Colletotrichum populi C.M. Tian & Zheng Li, **sp. nov.** Plate 3

MycoBank 564436

Lesions with black spots in circle and a light brown border, sometimes sunken. Differs from *Colletotrichum fructicola* by grey-white PDA colonies (reverse grey white or pale yellow) and from *C. gloeosporioides* by longer and irregularly lobed appressoria formed from conidia.

Type: China. Beijing, Shi Jingshan District, from leaves of *Populus nigra* var. *italica*, 25th October 2009, coll. C.M. Tian, HMBFU191 (**Holotype** BJFC, lyophilized PDA culture).

Etymology: *populi*, referring to the host species.

On leaves of poplar producing circular black spots with a dark brown border. Colonies on PDA at first white, becoming grey-white, reverse grey white or pale yellow, in 5 days at 28°C, daily growth 0.62–0.80 cm/day (mean = 0.73 ± 0.05, n = 5). Aerial mycelium cottony, white with few conidial masses, setae absent in culture. Conidia 9.8–17.7 × 3.0–7.3 μm (mean = 15.1 ± 2.0 × 5.2 ± 0.9, n = 30), conidia smooth-walled, hyaline, and cylindrical with obtuse ends. Appressoria 7.3–12.2 × 4.9–9.3 μm (mean = 9.4 ± 1.6 × 6.2 ± 1.2, n = 20), formed from conidia, brown to dark brown, irregularly lobed in shape.

Additional collections examined: **CHINA.** Beijing, Shi Jingshan District, from leaves of *Populus nigra* var. *italica*, 25th October 2009, coll. C.M. Tian, HMBFU161, HMBFU141, HMBFU173 (BJFC, lyophilized PDA cultures).

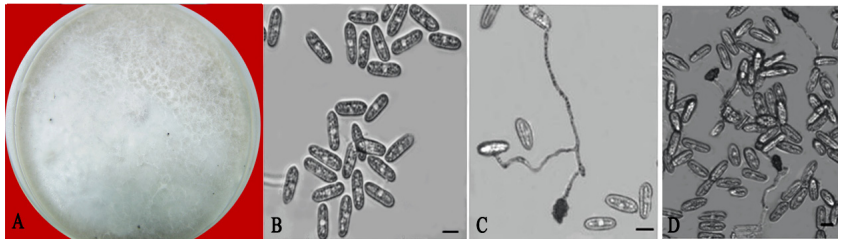

Plate 3. *Colletotrichum populi* isolates (Group 2): cultural and morphological characters of holotype (isolate 1-9-1). A: Colony; B: Conidia; C & D: irregular appressoria. Bars = 10 μm.

Comments: *Colletotrichum populi* can be separated from the other species by both morphological and molecular characters (Table 5). The similar *C. asianum* grows more slowly and produces narrower conidia (8.7–20.3 × 3–4.7 μm). The new species shares similar colony characteristics with *C. boninense*, *C. gloeosporioides,* and *C. kahawae*, all of which produce shorter and more rarely lobed appressoria. The genetically most closely related *C. fructicola* is distinguished by colonies that are grayish green in reverse with white edges. Colonies of *C. hymenocallidis,* which has similarly sized conidia and appressoria, appear denser in the central zone and produce fusiform conidia. *C. jasmine-sambac* differs by its colony with fimbriate margin.

Discussion

Both *Colletotrichum gloeosporioides* and *C. populi* have been found to cause anthracnose on poplars in Beijing region but can be distinguished by differing colony and appressoria morphologies as well as by molecular data derived from sequencing five genes. Appressorial size and shape have been used to distinguish some *Colletotrichum* species. For example, *C. gloeosporioides* was distinguished from *C. acutatum* by the shape of appressoria (Du et al. 2005). Crouch & Beirn (2009) pointed out that the size and shape of appressoria might be effective with the combination of host species during their work on anthracnose of cereals and grasses

Although rDNA-ITS gene is not an ideal marker for intraspecific relationships, it is very useful in many cases for reconstruction of interspecific relationships (Cai et al. 2009). Other gene sequences have been examined in the phylogenetic analysis of *Colletotrichum*. GS and calmodulin (CAL) were found to be useful when studying anthracnose on coffee berries (Prihastuti et al. 2009). Compared with six gene regions, GPDH, CAL and ACT were useful for inferring the relationship of *C. gloeosporioides* sensu lato (Cai et al. 2009). Whilst there is no defined set of genes to be used for taxonomic studies of *Colletotrichum*, molecular data from a wider range of genes would give better insights into this group of fungi.

Table 5. *Colletotrichum populi* and closely related species.

Species	Colony	Conidia (μm)	Appressoria (μm)	Reference
C. asianum	Greenish white; in center greyish to dark green	Cyl. with obtuse ends, 8.7–20.3 × 3–4.7	Circular to sl. irregular, 3–5.1 × 2.1–3.3	Prihastuti et al. 2009
C. boninense	White aerial mycelium, reverse cream to orange	Cyl., 11.5–17 × 4–7	Irregularly shaped, 6–17 × 4–15	Jouji et al. 2003
C. fructicola	White; in age grey at the center	Cyl. with ends obtuse to sl. rounded, 8.5–16 × 3.5–5	Ovoid, occ. clavate, oft complex, 6–9 × 5.5–7	Prihastuti et al. 2009
C. gloeosporioides	Greyish white to dark grey, aerial mycelium	Cyl., base truncate, 12–17 × 3.5–6	Clavate, ovate, obovate, 6–20 × 4–12	Sutton 1992
C. hymenocallidis	White; later pale grey with circles	Fusiform, straight, ends obtuse 14–20 × 5–6.5	Ovate, occ. clavate, 7–11 × 5–7.5	Yang et al. 2009
C. jasmine-sambac	White aerial mycelium, with fimbriate margin	Cyl., straight with obtuse ends, hyaline, 13–15 × 3.5–4	Shape variable, ovoid, clavate, sl. irregular, brown	Wikee et al. 2010
C. populi	White to grey, aerial mycelium	Cyl., straight with obtuse ends, 9.8–22.0 × 2.9–7.3	Irregular, edge lobed, 7.3–12.2 × 4.9–9.3	This research
C. kahawae	Grey, later grey to dark, olive. grey	Straight, cyl., guttulate, apex obtuse, 7.5–17 × 3.5–5	Circular to sl. irregular, 4.5–10 × 7.5	Prihastuti et al. 2009

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