Colletotrichum gloeosporioides sensu stricto Is a Pathogen of Leaf Anthracnose on Evergreen Spindle Tree (*Euonymus japonicus*)

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Abstract

Huang, L., Li, Q.-C., Zhang, Y., Li, D.-W., and Ye, J.-R. 2016. *Colletotrichum gloeosporioides* sensu stricto is a pathogen of leaf anthracnose on evergreen spindle tree (*Euonymus japonicus*). Plant Dis. 100:672-678.

The genus *Colletotrichum* is considered the eighth most important group of plant-pathogenic fungi in the world due to its scientific and economic importance. *Colletotrichum* spp. cause anthracnose disease in a wide range of economically important plants. *Euonymus japonicus* Thunb. (Celastraceae) is a broad-leaved evergreen tree that is widely planted in the parks and landscapes of China. An anthracnose occurs on

E. japonicus in China but there has been a disagreement on the identity of the fungal pathogen. In this study, the fungal isolate HYCG2-3 was determined by Koch's postulates to be a pathogen on *E. japonicus*. Based on the morphological and molecular methods, isolate HYCG2-3 was identified as *Colletotrichum gloeosporioides* sensu stricto within the *C. gloeosporioides* species complex.

The genus *Colletotrichum* Corda 1831 has a broad host range that includes over 3,200 species of mono- and dicotyledonous plants (Crous et al. 2004; Dean et al. 2012; O'Connell et al. 2012). Members of this genus cause major losses to economically important crops, especially fruit, vegetables, and ornamentals (Crouch et al. 2009; Kamle et al. 2013; Prusky 1996; Su et al. 2011). Its distribution is primarily in tropical and subtropical areas; however, a number of prominent species infect temperate crops (Cannon et al. 2012). These pathogens use a multistage hemibiotrophic infection strategy, which had emerged as a tractable model for studying fungal pathogenicity and plant immune responses (Huser et al. 2009; Narusaka et al. 2009; O'Connell et al. 2004; Perfect et al. 1999). The genus *Colletotrichum* is currently considered the eighth most important group of plant-pathogenic fungi in the world due to its scientific and economic importance (Dean et al. 2012).

Colletotrichum, an asexual genus, is linked to its sexual state, genus *Glomerella* Spauld. & H. Schrenk 1903 (Kirk et al. 2008; Ocfemia and Agati 1925; Shear and Wood 1907; Sutton 1980). According to the 1F=1N principle (namely, one fungus has only one name) adopted by the International Code of Nomenclature for algae, fungi, and plants (McNeill et al. 2012), *Colletotrichum* has priority over *Glomerella* and was chosen as the current name (Kirk et al. 2013). In spite of significant developments in fungal systematics in the last two decades, the taxonomy of genus *Colletotrichum* is far from fully solved (Dean et al. 2012). Uncertainties still exist over the systematics of the pathogens in this genus. The number of accepted species ranges from 11 in von Arx (1957), 22 in Sutton (1980), about 40 in Sutton (1992), and 60 in the Dictionary of the Fungi (Kirk et al. 2008). There are also 809 records in Index Fungorum and 883 epithets in MycoBank (Index Fungorum 2015; MycoBank 2015). The true number of accepted species still remains unknown. One of the most confusing species is *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. For example,

594 species of *Colletotrichum* were demoted by von Arx (1957) to synonyms of *C. gloeosporioides* sensu lato (s.l.), while Kirk et al. (2008) considered this species complex to have 600 synonyms. Based on morphological studies and phylogenetic analyses using actin (ACT), calmodulin (CAL), chitin synthase 1 (CHS-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and internal transcribed spacers (ITS), Weir et al. (2012) segregated the *C. gloeosporioides* species complex into 22 species and one subspecies.

Euonymus japonicus Thunb. (Celastraceae) is a broad-leaved evergreen tree widely planted as an ornamental in the parks and landscapes of China. Its red clusters of fruit among the green foliage are of high visual value in the fall. However, *Euonymus* spp. are often seriously damaged by insects and microorganisms (Yi et al. 2003). Anthracnose is one of the diseases caused by a fungus infecting the euonymus shrub. At present, there is a disagreement on the identity of the pathogen of this disease. Lee et al. (2005) considered that *C. boninense* was the pathogen of anthracnose. However, Wang et al. (1986) and Shang and Qiu (1992) attributed *C. gloeosporioides* in the anthracnose on *E. japonicus*. Because *C. gloeosporioides* is a species complex, the identity of the pathogen remains undetermined.

The objectives of this study were to (i) characterize the pathogen of anthracnose on evergreen spindle trees in Nanjing, China and (ii) determine the identity of the pathogen with both morphological and multilocus phylogenetic methods.

Materials and Methods

***Colletotrichum* isolation.** To isolate the fungus, infected leaves of *E. japonicus* with symptoms of anthracnose were randomly collected with a pair of sterile scissors from the campus of Nanjing Forestry University and the Xuan Wu Lake park in Nanjing, China in September 2014. These infected leaves were surface disinfested by immersion in 100% ethanol for 30 s and dried in sterilized petri dishes. Margins of the lesions were cut into small pieces (2 to 3 by 2 to 3 mm), placed on 2% potato dextrose agar (PDA) (five pieces per plate), and incubated at 28°C for 7 days in the dark.

Conidial masses were picked from sporulating colonies with a needle and pure cultures were obtained by monospore isolation using the technique of serial dilutions (Li et al. 2007). Monospore cultures

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Accepted for publication 8 September 2015.

<http://dx.doi.org/10.1094/PDIS-07-15-0740-RE>
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were transferred to new PDA plates and grown for further morphological and cultural characterization.

Pathogenicity tests. Ten purified fungal isolates were obtained as described above. Because these isolates shared almost identical morphological characteristics, strain HYCG2-3 was randomly selected for the pathogenicity assay and morphological description.

For Koch's postulates, HYCG2-3 was used to inoculate healthy leaves of *E. japonicus*. Fresh leaves of *E. japonicus* (15 days old) were used for pathogenicity testing. Conidia of HYCG2-3 were collected from 10-day-old cultures on PDA and suspended in sterile distilled water. The conidial suspensions were adjusted to concentrations of 10^5 , 10^6 , and 10^7 conidia ml^{-1} . The leaves were wounded with a red-hot needle 1 mm in diameter. Ten wounded leaves were inoculated with each spore concentration (10^5 , 10^6 , or 10^7 conidia ml^{-1}) using a pipette. Each concentration of conidia was inoculated to ≥ 10 leaves. After inoculation, the leaves were placed into petri dishes containing wet paper towel to retain the moisture and incubated at 25°C under a 12-h cycle of light and darkness. Leaves inoculated with the sterile distilled water were used as the negative control. To complete Koch's postulates, the fungus was reisolated from lesion margins according to the methods described above. The experiment was carried out three times and each treatment had three replicates.

Morphological analysis. Colony characteristics and pigment production on PDA were observed after 10 days at 28°C. Colony growth rates were measured on the day 7 and day 10. Conidia were collected from PDA after 10 days and mounted on glass slides in 85% lactic acid. Conidia size was measured according to methods described by Weir et al. (2012). Appressoria were induced to develop on the surface of hydrophobic glass plates in vitro. At least 30 measurements per structure were measured with a ZEISS Axio Imager A2m microscope using differential interference contrast illumination.

Molecular identification and phylogenetic analysis. Genomic DNA from the HYCG2-3 isolate was extracted using the method of Damm et al. (2008). The ITS, GAPDH, and partial sequences of the CHS-1 and ACT genes were amplified and sequenced using the primer pairs ITS-1F (Gardes and Bruns 1993) + ITS-4 (White et al. 1990), GDF1 + GDR1 (Guerber et al. 2003), CHS-354R + CHS-79F (Carbone and Kohn 1999), and ACT-512F + ACT-783R (Carbone and Kohn 1999), respectively. Polymerase chain reaction (PCR) was performed in an Eppendorf Nexus Thermal Cycler (Eppendorf) in a total volume of 25 μl . The ITS, GAPDH, CHS-1, and ACT PCR mixture contained 20 ng of genomic DNA, 0.2 μM each primer, and 12.5 μl of 2 $\times$ PCR Taq MasterMix (Applied Biological Materials Inc.). Conditions for PCR of these genes constituted an initial denaturation step of 3 min at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and a final denaturation step of 7 min at 72°C. DNA sequences obtained (forward and reverse) were aligned and edited with the Molecular Evolutionary Genetic Analysis (MEGA6.06) using Clustal W (Thompson et al. 1994). All the sequences generated in this study were deposited in GenBank.

Phylogenetic analysis was performed with each gene as well as the concatenated sequence alignment of four housekeeping genes (ITS, GAPDH, CHS-1, and ACTIN) using MEGA 6.0 software (Tamura et al. 2013). The sequences of 35 *Colletotrichum* isolates were obtained from GenBank for the analyses. Furthermore, the sequences of *C. boninense* and *C. hippeastri* were used as outgroups. A

multilocus phylogenetic tree was obtained using neighbor-joining analysis based on the Tamura three-parameter model with gaps complete delete option, and the tree was drawn with branch lengths measured in the number of substitutions per site. An interior-branch test using 1,000 bootstrap replications was performed to assess the relative stability of the branches.

Results

Symptom development. The disease development of anthracnose on the leaves of *E. japonicus* was observed in nature. In the early stage, there were some irregular chlorotic spots (0.5 to 1.8 mm in diameter, mean 0.98 mm) that occurred on the adaxial surface of the leaves (Fig. 1B). These spots enlarged and coalesced to form large, regular to irregular chlorotic to necrotic spots (Fig. 1C). Finally, most of the spots formed dry lesions and extensive necrotic areas (Fig. 1D). Fungal fruiting bodies (acervuli) developed on these lesions in concentric rings (Fig. 1E and F), typical symptoms of this disease. Leaves inoculated with water only remained symptom free (Fig. 1A).

Pathogenicity of fungal isolates. By day 2, hyphae were observed in the center of wounds on leaves inoculated with 10^7 conidia ml^{-1} . Typical disease symptoms appeared on day 3. By day 5, lesions were 3 to 4 cm in diameter and coalesced, and several acervuli were observed. On day 6, the necrotic areas had enlarged and contained numerous acervuli in concentric rings (Fig. 2). By day 8, surfaces of the inoculated leaves were wholly necrotic.

When the leaves were inoculated with 10^6 and 10^5 conidia ml^{-1} , disease development was similar but was slower than when inoculated with 10^7 conidia ml^{-1} (Fig. 2), and leaf death occurred on day 10 and day 13, respectively. A fungus with the same morphological characteristics as HYCG2-3 was consistently reisolated from the necrotic leaves. Leaves inoculated with double-distilled H_2O remained healthy.

Morphological characteristics of isolate HYCG2-3. After the HYCG2-3 was inoculated on PDA for 7 days, colonies of the agar produced strong brownish-orange pigmentation near the center (Fig. 3A). The average radial growth of the HYCG2-3 isolate on PDA was 8.2 mm day^{-1} . Aerial mycelia were dense, pale gray, and slightly felt-like (Fig. 3B). Conidia were hyaline, unicellular, smooth, and cylindrical. The ends of most conidia were obtuse but some were tapered toward the base ends, with some conidia tapering toward the basal end. Average conidial size was 15 to 20.2 by 5.1 to 6.7 μm , mean $\pm$ standard deviation = 17.4 ± 1.0 by 5.7 ± 0.3 μm , and length/width ratio = 3.1 (Fig. 3C).

Conidia germinated and developed appressoria on hydrophobic surfaces in vitro. Most conidia only developed an appressorium. Before germination, a medial septum developed, dividing the unicellular conidium into two cells of the same size (Fig. 4A). Germ tubes of different lengths grew from each cell of the conidium and produced pleurogenous, unicellular appressoria (Fig. 4A and B). A few conidia developed two appressoria. In this case, each cell of the two-celled conidium developed an appressorium (Fig. 4C and D). The appressoria developed either pleurogenously (Fig. 4C) or bipolarically (Fig. 4D). Most appressoria (92%) were pear shaped (Fig. 4E and F), and the rest were irregularly shaped (Fig. 4G through N). A few appressoria were two-celled (Fig. 4M and N). The average size of appressoria was 7 to 10.5 by 5 to 9.5 μm . Mature appressoria were darkly pigmented (Fig. 4). These morphological characteristics agree with the epitype of *C. gloeosporioides* sensu stricto (s.s.) (Cannon et al. 2008).

Fig. 1. Symptom development of anthracnose on the leaves of *Euonymus japonicus*. **A**, Healthy leaves. **B**, Chlorotic spots (0.5 to 1.8 mm in diameter, mean 0.98 mm) on diseased leaves. **C**, Coalesced chlorotic spots on diseased leaves. **D**, Dry, necrotic lesions and extensive necrotic patches on diseased leaves. **E**, Enlarged lesion with the typical concentric circles of acervuli. **F**, Typical concentric circles of acervuli.

The cuticle of HYCG2-3-infected leaves was removed and observed under the microscope after 7 days after inoculation. Compared with the hyphae that grew in dead leaf tissues, the apical cells of invading hyphae in the living cells seemed to be more coarse and less branched (Fig. 5A and B). Acervuli produced in infected tissues were subepidermal, typically with simple, short, erect conidiophores (Fig. 5C). Conidiogenous cells were hyaline and cylindrical, and most of them tapered toward the apex (Fig. 5D). Conidia in infected tissues were similar morphologically to those measured *in vitro*. Most appressoria were irregularly shaped, with dark-brown, thickened walls (Fig. 5E, i through xi).

Molecular characteristics of isolate HYCG2-3. The genes of ITS, CHS, GAPDH, and ACT from HYCG2-3 were sequenced and deposited in GenBank with the accession numbers KT033501 to KT033504. The ITS sequence of HYCG2-3 showed 100% similarity with a *C. gloeosporioides* from the GenBank database (accession JQ580527.1). The GAPDH sequence showed 100% similarity with *C. gloeosporioides* isolate CAUG15 (accession KP145401.1, GenBank). The CHS sequence showed 99% similarity with *C. gloeosporioides* isolate CAUG25 (accession KP1452382.1, GenBank). The ACT sequence showed 100% similarity with *C. gloeosporioides* strain C1014.6 (accession JX009462.1, GenBank).

The phylogenetic analysis of ITS sequences was performed with HYCG2-3, 35 ex-type strains, and authentic cultures of the *Colletotrichum* species complex, including *C. nupharicola*, *C. fructicola*, *C. aeschynomenes*, *C. alienum*, *C. musae*, *C. siamense*, *C. asianum*, *C. queenslandicum*, *C. salsolae*, *C. gloeosporioides*, *C. alatae*, *C. xanthorrhoeae*, *C. horii*, *C. aotearoa*, *C. psidii*, *C. kahawae* subsp. *ciggaro*, *C. theobromicola*, and *G. cingulata* “f. sp. *camelliae*” (Fig. 6). Among HYCG2-3, an ex-type strain of *C. gloeosporioides* s.s. (ICMP 17821) and six other authentic cultures of *C. gloeosporioides* s.s. strains formed a monophyletic lineage.

Phylogenetic analysis using the concatenated sequence alignment of four housekeeping genes (1,452 bp), including ITS, CHS, GAPDH, and ACT, also showed that HYCG2-3 and ex-type and authentic strains of *C. gloeosporioides* s.s. were monophyletic, with significantly higher bootstrap support for these groups (Fig. 7). These results indicated that HYCG2-3 belongs to *C. gloeosporioides* s.s. in the *C. gloeosporioides* complex.

Discussion

Colletotrichum spp. cause anthracnose in a wide range of economically important plants. More than one species of *Colletotrichum* can affect a single plant species (Noireung et al. 2012). *C. gloeosporioides*

Fig. 2. Lesion development of HYCG2-3 on detached leaves of *Euonymus japonicus* after inoculation by conidia for 6 days. **A**, Negative control with double-distilled H₂O. **B**, Leaves inoculated with a suspension of HYCG2-3 at 10^5 conidia ml⁻¹. **C**, Leaves inoculated with a suspension of 10^6 conidia ml⁻¹. **D**, Leaves inoculated suspension of 10^7 conidia ml⁻¹. **E**, Enlarged lesions on leaves inoculated 10^7 conidia ml⁻¹. Arrow indicates the concentric ring of fruiting bodies.

Fig. 3. Morphological characteristics of colonies and conidia of HYCG2-3. **A**, View of a colony showing pigmentation of the agar. **B**, View of a colony showing aerial hyphae and acervuli. **C**, Conidia. Scale bar = 5 μ m.

s.l. has been reported on *E. japonicus* in China, Japan, and the United States (Grand 1977; Kobayashi 2007; Parris 1959; Wu 1992). *C. griseum* Heald & F. A. Wolf, *C. boninense*, and a *Glomerella* sp. were also reported to cause anthracnose on *E. japonicus* in the United States, Korea, and Japan, respectively (Anonymous 1960; Hemmi 1918; Kobayashi 2007; Lee et al. 2005). Wu (1992) reported several species of *Colletotrichum* from *E. japonicus* in China, including *C. suttonii* W. P. Wu (nom. inval., Arts 39.1, 40.1 [Melbourne] due to no Latin diagnosis; Index Fungorum 2015), *C. crassipes* (Speg.) Arx, *C. griseum*, *C. gloeosporioides*, and *C. trichellum* (Fr.) Duke. In the past, *C. gloeosporioides* s.s. was not sufficiently typified and epitypification is necessary for

precise application of *C. gloeosporioides* s.s. Designating an epitype specimen with a separate living culture (ex-epitype) as well as the ITS sequence of ex-epitype was not done until 2008, when Cannon et al. (2008) epitypified this taxon to clarify the delineation of the species and subsequently stabilized the application of the name *C. gloeosporioides* s.s. Weir et al. (2012) studied the *C. gloeosporioides* complex, redelineated the species, and clarified the confusion using a multilocus approach. They accepted 22 species and one subspecies within *C. gloeosporioides* s.l. The true identities of the aforementioned members of *C. gloeosporioides* s.l. reported on *E. japonicus* remain unclear, however, and need to be further studied based on the latest developments by Cannon et al. (2008) and Weir et al.

Fig. 4. Appressoria of HYCG2-3 germinated in vitro. **A**, Appressorium formed from a conidium with a short germ tube. Arrow indicates a septum of the conidium. **B**, Appressorium developed from a conidium with a long germ tube. **C**, Two pleurogenous appressoria formed on a conidium. **D**, Bipolar appressoria on a single conidium. **E** and **F**, Pear-shaped appressoria. **G** through **N**, Irregularly shaped appressoria. Scale bars = 5 μ m.

(2012). At present, it is unclear whether the *C. gloeosporioides* s.l. reported by Wu (1992) from the Shijiazhuang of Hebei Provinces of Northern China is conspecific with HYCG2-3 (*C. gloeosporioides* s.s.) collected from Nanjing of Jiangsu Provinces in Southern China. Further research is needed to determine the exact identity of Wu's species. Weir et al. (2012) indicated that the black spot pathogen on paper mulberry reported from China by Yan et al. (2011) is *C. gloeosporioides* s.s. Comparison of the 430-bp length of the ITS region of HYCG2-3 and *C. gloeosporioides* s.s. (accession number HQ 634378 in GenBank) reported by Yan et al. (2011) indicates a 98.6% similarity.

The *C. boninense* species complex was studied by Damm et al. (2012) using multilocus molecular phylogenetic analysis (ITS, ACT, β -tubulin 2 (TUB2), CHS-1, GAPDH, histone (HIS3), and CAL) and segregated 18 species. Lee et al. (2005) sequenced 18S ribosomal DNA of *C. boninense* isolated from *E. japonicus* in South Korea and compared it with those in GenBank by BLASTing; however, they failed to provide an accession number for their sequence. Thus, the identity of this *C. boninense* in the species complex cannot be verified or determined.

In order to exactly distinguish the species of *Colletotrichum* from each other, morphological characteristics, including conidial size, shape, appressoria formation, sclerotia, setae, and acervuli, were used to describe some species of *Colletotrichum*. When *Colletotrichum* spp. infect host plants, infection occurs via an appressorium that develops from the germinating conidium on the plant surface, followed by turgor-driven penetration of the cuticle (Deising et al. 2000) and, in some cases, also of epidermal cells by infective hyphae (Bailey and Jeger 1992). Establishment within plant tissues is aided by host-induced virulence effectors delivered by appressoria and intracellular hyphae of the fungal pathogen (Kleemann et al. 2012; O'Connell et al. 2012). Therefore, appressoria are an important invading structure of *C. gloeosporioides*. In the present study, appressoria of *C. gloeosporioides* HYCG2-3 developed not only on hydrophobic glass surfaces but also in the living tissues of *E. japonicus*. However, the morphological characteristics of appressoria were variable. When appressoria formation was induced on hydrophobic surfaces in vitro, most appressoria were pear shaped and only a few were irregular. When leaves of *E. japonicus* were inoculated with HYCG2-3, the appressoria of *C. gloeosporioides* were mostly irregular in shape. These results suggested that the shape of appressoria is variable in different environments and not a stable taxonomic characteristic. For morphological identification, the shape of appressoria in vitro was more reliable.

The ITS sequence is a popular target for molecular characterization of eukaryotic microbes (Johannesson and Stenlid 1999; Malan et al. 2011). The ITS region in these microbes was used to develop a molecular marker for species identification. ITS has been adopted as a universal DNA barcode marker for fungi (Schoch et al. 2012) but, for some large fungal genera such as *Colletotrichum* and *Alternaria*, their species cannot be effectively differentiated solely using ITS sequence data (Brun et al. 2013; Weir et al. 2012). Thus, other

genes are also used to differentiate among species, including ACT, HIS, CHS, and GAPDH (Cannon et al. 2012). In order to distinguish the different species of the *C. gloeosporioides* complex, the phylogenetic tree calculated from the alignment of concatenated sequences of ITS, GAPDH, CHS, and ACT was analyzed (Weir et al. 2012). In the current study, based on the analyses of the phylogenetic trees generated from the ITS sequences alone and concatenated sequences of ITS, ACT, CHS, and GAPDH, HYCG2-3 and ex-type and authentic cultures of *C. gloeosporioides* s.s. were clustered in the same clade with high bootstrap values (Fig. 7).

Fig. 6. Phylogenetic tree of HYCG2-3 with allied taxa calculated from internal transcribed spacer using neighbor-joining method. Bootstrap values >60% (1,000 replications) are given at the nodes. Ex-type and authentic cultures are emphasized in bold. *Colletotrichum boninense* and *C. hippeastri* isolates are used as outgroups. Bar = 0.005 substitutions per nucleotide position.

Fig. 5. Morphological characteristics of HYCG2-3 in infected leaves. A, Mycelium of HYCG2-3 in living leaf tissues. B, Mycelium of HYCG2-3 in dead tissues. C, Acervuli and conidiophores of HYCG2-3. D, Conidiogenous cells and conidium. E i through xi, Variability of appressoria of HYCG2-3 in infected leaf tissue. Scale bars = 5 μ m.

Fig. 7. Phylogenetic tree of HYCG2-3 with allied taxa calculated from concatenated sequences of ITS, GAPDH, CHS, and ACT using neighbor-joining method. bootstrap values >60% (1,000 replications) are given at the nodes. Ex-type and authentic cultures are emphasized in bold front. *Colletotrichum boninense* and *C. hippeastri* isolates are used as outgroups. Bar = 0.01 substitutions per nucleotide position.

In conclusion, *C. gloeosporioides* s.s. was identified as a pathogen causing anthracnose on *E. japonicus* in Nanjing, China in our study.

Acknowledgments

This work was financially supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We thank J. LaMondia for reviewing the manuscript.

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