Botrytis polyphyllae: A New *Botrytis* Species Causing Gray Mold on *Paris polyphylla*

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Abstract

Paris polyphylla is an important perennial medicinal plant in China. A disease similar to gray mold on *P. polyphylla* occurred at the seedling stage in March 2016 and 2017 in Tengchong city, Yunnan Province of China. The disease resulted in up to 50% mortality in serious cases. Isolates from diseased plants grew 10.6 mm/day at 20°C on PDA. After 21 days, sclerotia were spherical to elliptical (0.4–2.5 × 0.3–1.8 mm). Conidia from diseased tissues were hyaline to pale brown, long, ovoid, unicellular, and measured 15.1–24.5 × 8.8–13.4 µm; conidiophores were

526–1,064 × 12–15 µm. Isolates did not form conidiophores or conidia on PDA or MYA. A phylogenetic analysis based on G3PDH, RPB2, and HSP60 sequence data supported assignment of three representative isolates as a new species of *Botrytis*. Based on morphological, phylogenetic characteristics and Koch's Postulates, the causal agent of gray mold on *P. polyphylla* was identified as a novel species, *Botrytis polyphyllae*.

Keywords: *Paris polyphylla*, gray mold

Paris polyphylla (family Trilliaceae) is an important perennial medicinal plant, and its dried rhizome is used in traditional Chinese medicine for its anti-inflammatory and hemostatic effects (Zhao et al. 2014). The demand for the rhizome is increasing by 20% every year, and approximately 3,000 tons were consumed in China in 2012 (Yang et al. 2012). The cultivation area of *P. polyphylla* is becoming larger as wild *P. polyphylla* is very limited. During March of 2016 and 2017 in Tengchong City, Yunnan Province of China, seedlings of *P. polyphylla* developed symptoms of gray mold. Infected leaves and petioles were water-soaked and rotted. Stem infections resulted in damping-off. Up to 50% of the plants were affected. During periods of high humidity, lesions became covered with gray mold; morphology of conidia and conidiophores were consistent with those of *Botrytis*.

The genus *Botrytis* is the focus of intensive scientific research worldwide. It can infect more than 1,400 species of cultivated plants, many of which are important agricultural crops, such as strawberries and table grapes (Fillinger and Elad 2015). The genus *Botrytis* has been divided into 22 species based on Hennebert's taxonomy (Hennebert 1973). In the last decade, at least 10 new species have been identified based on their morphological and molecular characteristics (Ferrada et al. 2016; Garfinkel et al. 2017; Grant-Downton et al. 2014; Li et al. 2012; Saito et al. 2016; Walker et al. 2011; Zhang et al. 2010a, b, 2016; Zhou et al. 2014).

To date, the identification of *Botrytis* spp. has mainly relied on morphological characteristics such as colony morphology, conidial shape and size, and the organization and size of the sclerotia (Hennebert 1973). However, morphological features are usually

inconsistent among different *Botrytis* species or isolates. Currently, DNA-based molecular techniques have been developed and are widely used for fungal identification. These include DNA sequencing of internal transcribed spacer regions (ITS) (White et al. 1990) and other protein coding sequences. Phylogenetic analyses using a single gene or a multilocus analysis generated from loci such as glyceraldehyde-3-phosphate dehydrogenase (G3PDH), heat shock protein 60 (HSP60), and the second-largest subunit of DNA-dependent RNA polymerase II (RPB2) genes have been used to support morphological characteristics (Staats et al. 2005).

The pathogen of gray mold on *Rhizoma paridis* (*Paris polyphylla*) leaves was reported to be *B. cinerea* in Hubei Province, China (You et al. 2014). In the present study, symptoms of diseased plants found in Yunnan Province, China shared similarities with gray mold. However, the cultural characteristics of the isolates were different from those of *B. cinerea*. The aim of this study was to identify the causal agent and describe the species of *Botrytis* obtained from *P. polyphylla* based on morphological characteristics and multigene molecular phylogeny, and to assess pathogenicity to several fruits and dicotyledonous plants.

Materials and Methods

Fungal isolation. Samples of diseased *P. polyphylla* were collected in Yunnan Province, China in March 2016 and 2017. To obtain cultures, diseased tissue pieces were soaked in 10 ml of a 3% NaOCl solution for 1 min, washed with sterile distilled water (SDW) three times, and subsequently plated on potato dextrose agar (PDA) (200 g of potato, 20 g of glucose, and 20 g of agar per liter). Cultures were incubated at 20°C in the dark for 48 h and then transferred to new plates of PDA. Isolates were purified by transferring hyphal tips to PDA, incubation at 20°C, and stored in PDA tubes at 4°C.

Morphological and biological characteristics. Three isolates (YN1, YN2, and YN3) were selected for morphological and biological characterization. To observe colony morphology, mycelial plugs were transferred from the edge of 5-day-old colonies onto fresh PDA plates and incubated at 20°C in the dark. Thirty sclerotia were randomly chosen from each isolate for measurement after 21 days. To enhance sporulation, mycelial plugs were inoculated onto both PDA and malt yeast extract agar (MYA) (10 g of malt extract, 4 g of glucose, 4 g of yeast extract, and 15 g of agar per liter) and placed under near-UV light.

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

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Table 1. Origin and GenBank accession numbers of the sequences for three *Botrytis* isolates used in this study

Species	Isolate	Host	Location	GenBank accession no.		
				RPB2	HSP60	G3PDH
<i>B. polyphyllae</i>	YN1	<i>Paris polyphylla</i>	Tengchong	MG431975	MG431978	MG387957
<i>B. polyphyllae</i>	YN2	<i>P. polyphylla</i>	Tengchong	MG431976	MG431979	MG387958
<i>B. polyphyllae</i>	YN3	<i>P. polyphylla</i>	Tengchong	MG431977	MG431980	MG387959

Fig. 1. Colonial morphology of YN3 on PDA at 20°C after incubation for 6 days, 12 days, and 21 days. The mycelium was pale gray to brown; the abundant sclerotia were light brown to black; no conidia and conidiophores produced.

Fig. 2. Morphological characters of conidiophores and conidia of YN3 from the diseased plant. **A** and **C**, Conidiophores were straight, septate, pale brown, branching alternate or whorled near the top. **B**, The conidia were hyaline to pale brown, long ovoid, unicellular. Bars = 20 μ m.

To determine the optimum temperature for colony growth of the isolates, mycelial plugs (5 mm diameter) were plated on new fresh PDA plates (90 mm diameter) and incubated at 5, 10, 15, 20, 25, 30, and 35°C. Each of the three isolates was tested at all 7 temperatures with 5 replicates per isolate. Colony diameter was measured after 5 days of incubation. The colony growth rate was calculated by diameter and expressed as millimeters per day.

DNA extraction, PCR amplification, and sequencing. Genomic DNA was extracted from 3 isolates by the CTAB method according to Gawel and Jarret (1991) with a slight modification. Four loci including ITS, G3PDH, RPB2, and HSP60 were amplified with the primer pairs ITS1/ITS4 (White et al. 1990), G3PDHfor/G3PDHrev, RPB2for/RPB2rev, and HSP60for/HSP60rev (Staats et al. 2005), respectively. PCR amplification protocols were performed as previously described (Staats et al. 2005; White et al. 1990). The amplified products of each gene were separated by agarose gel (1%, wt/vol) electrophoresis and sequenced by TSINGKE, Beijing, China with a 3730 DNA Analyzer (Applied Biosystems, U.S.A.). Sequences obtained from forward and reverse primers were analyzed with the BioEdit sequence alignment editor 7.1.11 to obtain consensus sequences (Hall 1999) and were submitted to GenBank (Table 1).

Phylogenetic analysis. Partial gene sequences of G3PDH, RPB2, and HSP60 were used to establish a concatenated dataset (G3PDH + RPB2 + HSP60) and three individual datasets (G3PDH, RPB2, and HSP60). The phylogenetic analysis included the 3 isolates collected in this study and reference isolates representing 35 species of *Botrytis* (Garfinkel et al. 2017; Grant-Downton et al. 2014; Li et al. 2012; O’Gorman et al. 2008; Rupp et al. 2017; Saito et al. 2014, 2016; Staats et al. 2005; Zhang et al. 2010a, b, 2016; Zhou et al. 2014). Strain 9201 of *Monilinia fructigena* and strain 484 of *Sclerotinia sclerotiorum* (Staats et al. 2005) were used as outgroups (Supplementary Table S1). *Botrytis mali* was deleted from the combined dataset and the separate HSP60 and RPB2 datasets, because the HSP60 and RPB2 gene sequences were absent. Maximum likelihood (ML) trees were computed in MEGA 6.0 (Tamura et al. 2013). The pairwise alignment parameters were gap opening penalty 15 and gap extension penalty 6.66. The multiple alignment parameters were gap opening penalty 15, gap extension penalty 6.66, the delay divergent cutoff 30%; DNA weight matrix IUB, transition weight 0.5, negative matrix off, and delay divergent cutoff 30%. All positions containing gaps were excluded from the analysis. The bootstrap consensus tree was inferred from 1,000 replicates. Branches corresponding to partitions reproduced in <50% bootstrap replicates were collapsed. The alignments and trees were deposited in TreeBASE (S23565, S23566, S23567, S23568).

Pathogenicity tests. Isolate YN3 was used to inoculate detached leaves and stems of *P. polyphylla* seedlings. Five stem segments injured with a sterilized insect needle 1 mm deep and 10 uninjured leaves were placed on a plastic tray. Mycelial plugs (3 mm diameter) cut from the edge of a 5-day-old colony were transferred onto the stem segments and leaves individually. Five stem segments injured

as above and 10 uninjured leaves were treated with PDA plugs (3 mm diameter) alone and used as controls. High relative humidity was maintained by covering the tray with plastic wrap. The tray was incubated at 20°C in the dark for 24 h and then in 12 h light/12 h dark for 7 days.

Pathogenicity to fruits of strawberry (*Fragaria × ananassa*), cherry tomato (*Solanum lycopersicum*), cherry (*Cerasus pseudocerasus*), table grape (*Vitis vinifera*), and cucumber (*Cucumis sativus*) was determined with isolate YN3. Ten fruits of each plant (cherry tomato, cherry, table grapes, and cucumber) injured with a 3-mm-diameter puncher and 10 uninjured fruits (strawberry) were inoculated with 5-day-old mycelial plugs (3 mm diameter). Ten fruits of the four plants injured as above and 10 uninjured strawberry fruits were inoculated with PDA plugs (3 mm diameter) to serve as controls. The incubation conditions were the same as above.

Disease incidence (%) was calculated as the percentage of host tissues (leaves, stem segments, or fruits) with lesions among the total number of the leaves (stems segments or fruits) inoculated with the isolate.

Results

Taxonomic description. *Botrytis polyphyllae* Shan Zhong & Guo-Zhen Zhang, *sp. nov.* (Figs. 1–2)

Mycobank: MB823456

Holotype: China, Yunnan Province, Tengchong City, 25°03’N, 98°50’E, dried culture of YN3 isolated from a diseased leaf of *Paris polyphylla*, 29 Mar 2017, G. Z. Zhang, holotype HMAS 247741, ex-type culture CGMCC 3.18842.

Etymology: *polyphyllae*, refers to the host species, *polyphylla*.

Descriptions: Colonies grew on PDA at an average rate of 10.6 mm/day at 20°C. Colonies were pale gray to brown with abundant sclerotia (Fig. 1), but no conidia or conidiophores were formed

Fig. 3. Average colony growth rate of three isolates on PDA at 5 to 35°C. The bar represents the standard errors of the means ($n = 15$).

Table 2. Colony growth rate and morphological characteristics of selected *Botrytis* spp.

Species	Isolate	Growth ^a (mm/day)	Sclerotia		Reference
			Shape ^b	Size (mm)	
<i>B. polyphyllae</i>	YN3	10.6	I, OE, S	0.4–4.5 × 0.3–2.2	This study
<i>B. sinoviticola</i>	GBC-5	13	I, OE, S	1.0–2.2 × 1.0–2.0	Zhou et al. 2014
<i>B. californica</i>	X503	13.5	I, OE, S	0.6–4.2 × 0.6–2.3	Saito et al. 2016
<i>B. calthae</i>	N.D. ^c	N.D.	I, OE, S	3.4–5.5 × 0.2–3.4	Hennebert and Groves 1963
<i>B. pseudocinerea</i>	N.D.	N.D.	I, OE, S	2.2–5.5 × 1.1–3.4	Walker et al. 2011
<i>B. fabae</i>	BC-22	13	I, OE, S	0.4–3.2 × 0.3–2.1	Zhang et al. 2010a
<i>B. pelargonii</i>	N.D.	N.D.	HS	2.5–4.8 × 1.9–2.4	Mirzaei et al. 2008
<i>B. cinerea</i>	BC-1	17	I, OE, S	0.7–27.5 × 0.5–12	Zhang et al. 2010a

^a Growth = Colony growth rate (PDA, 20°C).

^b Shape of sclerotia: HS = hemispherical, I = irregular, OE = oblong/elliptical, S = spherical.

^c N.D. = not determined.

on PDA and MYA even under near-UV light; sclerotia were solitary to aggregated, dark brown to black, irregular, spherical to elliptical, $0.4\text{--}2.5 \times 0.3\text{--}1.8$ mm (av. 1.5×1.0 mm, $n = 30$) (Fig. 1). Conidiophores from diseased plants were straight, septate, pale brown, branching alternate or whorled near the top, $526\text{--}1,064 \times 12\text{--}15$ μm (Fig. 2A); conidia from diseased plants were hyaline to

pale brown, long ovoid, unicellular, $15.1\text{--}24.5 \times 8.8\text{--}13.4$ μm (av. 21.1×11.3 μm , $n = 50$) (Fig. 2B) (Table 2).

The optimum temperature for colony growth on PDA was 20°C ; no growth occurred above 30°C (Fig. 3). This relatively low temperature may reflect an adaption to the cool environment of Yunnan Province.

Fig. 4. Molecular phylogeny of *Botrytis* species and outgroups *Monilinia fructigena* and *Sclerotinia sclerotiorum* presented in a maximum-likelihood tree inferred from the dataset containing partial DNA sequences of G3PDH. The numbers at each node indicate the bootstrap percentage ($n = 1,000$). Bootstrap support values less than 50% are not shown. Isolates of YN1, YN2, and YN3 were collected in this study.

Fig. 5. Molecular phylogeny of *Botrytis* species and outgroups *Monilinia fructigena* and *Sclerotinia sclerotiorum* presented in a maximum-likelihood tree inferred from the dataset containing partial DNA sequences of RPB2. The numbers at each node indicate the bootstrap percentage ($n = 1,000$). Bootstrap support values less than 50% are not shown. Isolates of YN1, YN2, and YN3 were collected in this study.

Phylogenetic analysis. Only two nucleotide differences in 418 base pairs of the partial ITS sequences of *B. cinerea* (AM235297), *B. pseudocinerea* (JN692379), *B. pelargonii* (AJ716290), *B. fabae* (AJ716303), and *Botrytis* sp. were seen in this study. The ITS sequences do not have sufficient resolution for the phylogenetic analysis (Saito et al. 2016; Zhang et al. 2016).

Fig. 6. Molecular phylogeny of *Botrytis* species and outgroups *Monilinia fructigena* and *Sclerotinia sclerotiorum* presented in a maximum-likelihood tree inferred from the dataset containing partial DNA sequences of HSP60. The numbers at each node indicate the bootstrap percentage ($n = 1,000$). Bootstrap support values less than 50% are not shown. Isolates of YN1, YN2, and YN3 were collected in this study.

The sequences of the three representative isolates in this study (Table 1), as well as those from other *Botrytis* species and outgroups (*M. fructigena* and *S. sclerotiorum*) obtained from GenBank were used for a phylogenetic analysis. The DNA sequences of G3PDH, RPB2, and HSP60 were used to establish three separate datasets and a combined dataset. Individual alignment datasets for each

Fig. 7. Molecular phylogeny of *Botrytis* species and outgroups *Monilinia fructigena* and *Sclerotinia sclerotiorum* presented in a maximum-likelihood tree inferred from the dataset containing partial DNA sequences of G3PDH, RPB2, and HSP60. The numbers at each node indicate the bootstrap percentage ($n = 1,000$). Bootstrap support values less than 50% are not shown. Isolates of YN1, YN2, and YN3 were collected in this study.

marker consisted of 83 sequences for G3PDH, 81 for RPB2, and 81 for HSP60. The combined dataset consisted of 81 fungal taxa. Individual gene analyses and the combined gene analysis (G3PDH + RPB2 + HSP60) resulted in similar tree topologies (Figs. 4–7).

The results of the concatenated dataset showed that members of the genus *Botrytis* formed three subclades (I, II, and III) (Fig. 7). Eight *Botrytis* species, *B. californica*, *B. calthae*, *B. cinerea*, *B. fabae*, *B. pelargonii*, *B. pseudocinerea*, *B. sinoviticola*, and *Botrytis* sp. in this study formed subclade II with 100% bootstrap support. Our *Botrytis* sp. was recognized as a separate taxon in the phylogenetic tree, and was most closely related to *B. cinerea*, *B. pelargonii*, and *B. fabae*.

Pathogenicity. Leaves of *P. polyphylla* inoculated with the new *Botrytis* species developed light brown spots with a water-soaked halo 3 days after inoculation (Fig. 8C). After 7 days, lesions were

water-soaked and rotted with 80% incidence (Fig. 8D). Stem segments developed fusiform lesions 7 days after inoculation with 100% incidence (Fig. 8E). No symptoms were observed on the non-inoculated control leaves and stems. Koch's Postulates were fulfilled by reisolating the same pathogen from the diseased tissues.

The fruits of strawberry, cherry tomato, and cherry inoculated with mycelial plugs showed rot 4 days postinoculation (Fig. 8F–H), and no symptoms were observed on the table grapes and cucumber and noninoculated control fruits (not shown).

Discussion

This study describes a new species of *Botrytis*, *B. polyphyllae*, on the basis of multilocus phylogenetic analyses and morphological characteristics that facilitate their distinction from other *Botrytis* species. Our results, which were based on the phylogenetic analyses of

Fig. 8. Symptoms of *Botrytis polyphyllae* on various hosts. **A** and **B**, *Paris polyphylla* infection in the field: diseased plants showed damping-off, white to gray mold appeared at the base of the stem; the leaves were water soaked, and dark brown to black rot was present on the petiole base. **C** to **E**, Pathogenicity of isolate YN3 to detached noninjured leaves and injured stem segments from *P. polyphylla*: the leaves showed light brown spots and the tissues became hyaline around the spots after 3 days and the spots turned brown to black after 7 days; stems showed fusiform lesions after 7 days. **F** to **H**, Pathogenicity of isolate YN3 to strawberry, cherry tomato and cherry fruits: strawberry developed a gray rot, cherry tomato and cherry fruit rotted after 4 days.

four datasets, indicated that *Botrytis* species fall into three subclades (Fig. 7). The newly described taxon, *B. polyphyllae*, was a separate species clustered with high bootstrap support and was more closely related to *B. cinerea*, *B. pseudocinerea*, *B. pelargonii*, and *B. fabae* in subclade 2.

B. polyphyllae formed conidia and conidiophores on *P. polyphylla* but did not sporulate on PDA or MYA in this study. This phenotype was also found in another *Botrytis* species, *B. caroliniana*, which was in the subclade III based on the molecular data and isolated from blackberry (Li et al. 2012). The morphological characteristics supported the segregation of *B. polyphyllae* from its closest phylogenetic sister taxa. The sclerotia of *B. polyphyllae* were 0.4–2.5 × 0.3–1.8 mm, and they were smaller than those of most other *Botrytis* species (Table 2).

Botrytis species are divided into two subclades in the phylogenetic tree by the partial genes (G3PDH, RPB2, and HSP60) (Staats et al. 2005). The subclade containing *B. cinerea* infects dicotyledons in general, while the subclade containing *B. aclada* infects monocotyledons. In this study, *B. polyphyllae* clustered to the same subclade with *B. cinerea*. *B. polyphyllae* infects *P. polyphylla* in nature, and by artificial inoculation, the fruit of strawberry, cherry tomato, and cherry, but not table grapes or cucumber. An extensive investigation should be carried out to determine the host range of *B. polyphyllae*. *B. cinerea* has been reported to cause gray mold on the foliage of *R. paridis* (*P. polyphylla*) in Hubei Province, China (You et al. 2014). To our knowledge, this is the first report of gray mold caused by the newly described *B. polyphyllae* on *P. polyphylla* in Yunnan Province, China.

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