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RESEARCH NOTE

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Identification and Characterization of *Gonatobotryum apiculatum* Causing Leaf Spot and Blight on *Sinowilsonia henryi*

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ABSTRACT

Sinowilsonia henryi is a rare and endangered plant, as well as an endemic species in China. In July 2018, leaf spot and blight disease was observed on *S. henryi* in Yichang, Hubei, China. A fungus isolated from disease tissues was identified as *Gonatobotryum apiculatum* based on morphology and sequence analyses of ITS and LSU regions. Phylogenetic analyses indicated that the species belongs to Dothioraceae (Dothideales). Morphologically, the species produced two distinct types of conidia from authentic media, both conidia were described here. Pathogenicity tests showed that the fungus is a pathogen causing leaf spots on *S. henryi*. This is the first report of leaf spot and blight disease on *S. henryi* caused by *G. apiculatum* in China.

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Sinowilsonia henryi Hemsl. is the only species of the monotypic genus *Sinowilsonia* (Hamamelidaceae) [1]. It is also a threatened and endangered plant endemic to China, which is included in the list of Chinese Dangerous Plant Red Paper (category II) [2]. As a relict species of the Tertiary period, this plant is considered to be an ideal material for the occurrence and evolution of angiosperms [3,4]. The plant is also used as an ornamental for its flourish leaves and beautiful tree shape [5].

Previous studies on *Sinowilsonia henryi* were mainly focused on its systematics [6,7], reproductive biology [8] and genetic diversity [9]. No any disease has been reported on this species. In July 2018, leaf spot and blight were observed on the plant in Dalaoling National Forest Park (31°02′38″N, 110°57′17″E), Yichang, China. Approximately 30% of the leaves on the tree were infected. Some small round or irregular necrotic spots occurred on the leaves. The edge of the spot was brown and the center turned to gray. Severely, brown lesions extended and large areas of the leaf were withered (Figure 1(A)). The aim of this study is to isolate and identify the causal agent based on morphological characterization and sequence analyses.

Diseased leaves were cut into small pieces, then placed into Petri dishes with moist filter papers and incubated at 25 °C in darkness. A kind of uncommon conidia with erect conidiophore was developed from the tissues after 1 day incubation. Conidia from a single conidiophore were picked up using sterile glass needles under a stereomicroscope and transferred onto potato dextrose agar (PDA). Four pure cultures were obtained and deposited in the Culture Collection of Yangtze University (YZU).

Genomic DNA of the fungus (YZU 181227) was extracted from mycelia using the method of Cenis [10]. Two gene regions including the internal transcribed spacer (ITS) and large subunit (LSU) of rDNA were amplified with the primer pairs ITS5/ITS4 [11] and LROR/LR5 [12], respectively. The PCR amplification was performed in a 25 μL reaction mixture, which contained 12.5 μL 2×*Taq* PCR StarMix, 2 μL DNA template, 1.25 μL of each primer and 8 μL ddH_2O. Successful PCR products were purified and sequenced by BGI (Beijing Genomics Institute, China). Based on the results of BLASTn searches, the two gene sequences were 99% identity to those of *Gonatobotryum apiculatum* (CBS 182.68). To know the phylogenetic position of the strain YZU 181227, reference sequences were downloaded from NCBI database according to the BLASTn results and relevant publication [13]. The sequences were aligned with ClustalW and edited in Mega v.7.0 [14]. Phylogenetic trees based on the ITS and LSU gene sequences were constructed using maximum likelihood method with 1000 bootstrap replications in RAxML v.7.2.8 [15]. *Catinella*

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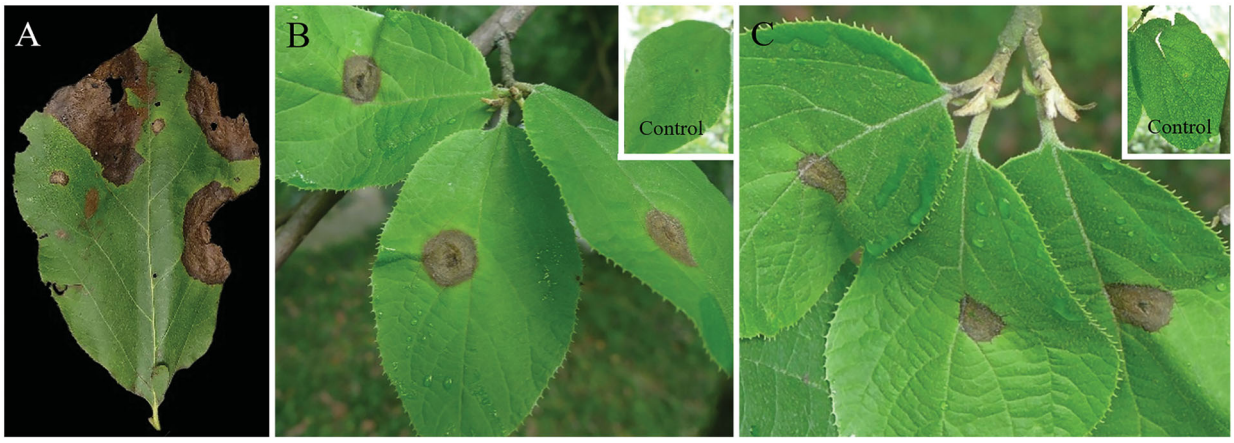

Figure 1. Natural symptoms on *Sinowilsonia henryi* (A); pathogenicity tests on wounded leaves of living host plants after 7-day inoculation of mycelium blocks (B) and spore suspension (C).

Table 1. Strains and their accession numbers used in the phylogenetic analyses.

		Accession numbers	
Species	Strain	ITS	LSU
Aureobasidium pullulans	CBS 584.75	KT693733	DQ470956
Catinella olivacea	UAMH 10679	DQ915483	EF622212
Columnosphaeria fagi	CBS 171.93	KT693737	AY016359
Delphinella strobiligena	CBS 735.71	MH860318	DQ470977
Dothidea hippophaës	CBS 188.58	MH857750	DQ678048
Dothidea insculpta	CBS 189.58	AF027764	DQ247802
Dothidea sambuci	DAOM 231303	AY883094	AY544681
Dothiora cannabinae	CBS 737.71	MH86032	DQ470984
Dothiora elliptica	CBS 736.71	KU728502	GU301811
Elsinoë centrolobi	CBS 222.50	MH856595	DQ678094
Elsinoë phaseoli	CBS 165.31	MH855166	DQ678095
Elsinoë veneta	CBS 150.27	KX887282	DQ767658
Endosporium aviarium	UAMH 10530	EU304350	EU304351
Endosporium populi-tremuloidis	UAMH 10529	EU304347	EU304348
Gonatobotryum apiculatum	CBS 182.68	MH859103	MH870816
Gonatobotryum apiculatum	SX1	KJ620838	–
Gonatobotryum apiculatum	**YZU 181227**	**MK895978**	**MK895979**
Kabatiella caulivora	CBS 242.64	KT693740	EU167576
Myriangium duriaei	CBS 260.36	MH855793	DQ678059
Myriangium hispanicum	CBS 247.33	MH855426	GU301854
Phaeocryptopus nudus	CBS 268.37	EU700371	GU301856
Phaeosclera dematioides	CBS 157.81	MH861313	GU301858
Stylodothis puccinioides	CBS 193.58	KY929139	AY004342
Sydowia polyspora	CBS 116.29	MH855019	DQ678058

olivacea UAMH 10679 was selected as an outgroup. Sequences of the strain YZU 181227 were deposited in the NCBI database with accession numbers shown in Table 1. Phylogenetic analysis of ITS region indicated that strain YZU 181227 fell into a subclade of *Gonatobotryum apiculatum* with strains CBS 182.68 and SX1. The present strain clustered with CBS 182.68 was supported by a bootstrap value of 95% (Figure 2), also supported by a bootstrap value of 91% in the phylogenetic analysis of LSU region (Figure 3). Furthermore, both phylogenetic analyses exhibited the same result that *Gonatobotryum apiculatum* should reside in the family Dothioraceae sensu lato (Dothideales), which was unclear from previous references and MycoBank (www.mycobank.org).

To observe the colony characteristics of the species, mycelial plugs (6 mm diam) from the edge of 3-day-old colonies were transferred to the center of PDA. After incubated at 25 °C for 7 days in darkness, colonies were fawn, grayish sepia in reverse side, 32–33 mm diam. (Figure 4(A)). Micro-morphology was determined on the host, PDA and water agar (WA). Sporulation structures and conidial morphology were determined in sterile distilled water and photographed using a Nikon ECLIPSE Ni-U microscope system (Nikon, Tokyo, Japan). All the four obtained strains exhibited the same feature. On the host, conidia produced from the terminal (mostly) or intercalary conidiogenous ampullae of conidiophores (Figure 4(B)). Conidiogenous ampullae showed cicatrized scars as distinctly echinulate (Figure 4(C)). On PDA, conidiophores were erect or flexuous, septate, unbranched, nodose (1–4), 215–700 μm long and 7–11 μm wide. Conidia from the conidiophores were catenate, aseptate, smooth, pale to brown, ellipsoidal to globose, 4.5–12 × 3–6 µm in size ($n = 100$) (Figure 4(D–F)). Based on above morphological descriptions, the fungus was identical to *Gonatobotryum apiculatum* [16]. Meanwhile, numerous conidia were gathered on the base of colony on PDA showing pus-like heap. The conidia were transparent, oval to globose, aseptate, 5–11 × 3.5–5 µm in size ($n = 100$) (Figure 4(G)). Its sporulation pattern could be clearly observed on WA after 2 days (Figure 4(H)). All those conidia were directly produced from hyphae on the surface of the medium. This is the first description of this type of conidia from *G. apiculatum*. Jacob and Bhat [17] reported two types of conidia of *G. bimorphosporum*. However, it was described that later formed conidia (the second type) arise from first-formed conidia, which totally different from the present description (*G. apiculatum*).

Pathogenicity of the fungus was tested on leaves of living plant of *Sinowilsonia henryi* by inoculating with mycelia plugs and spore suspension. Inoculated

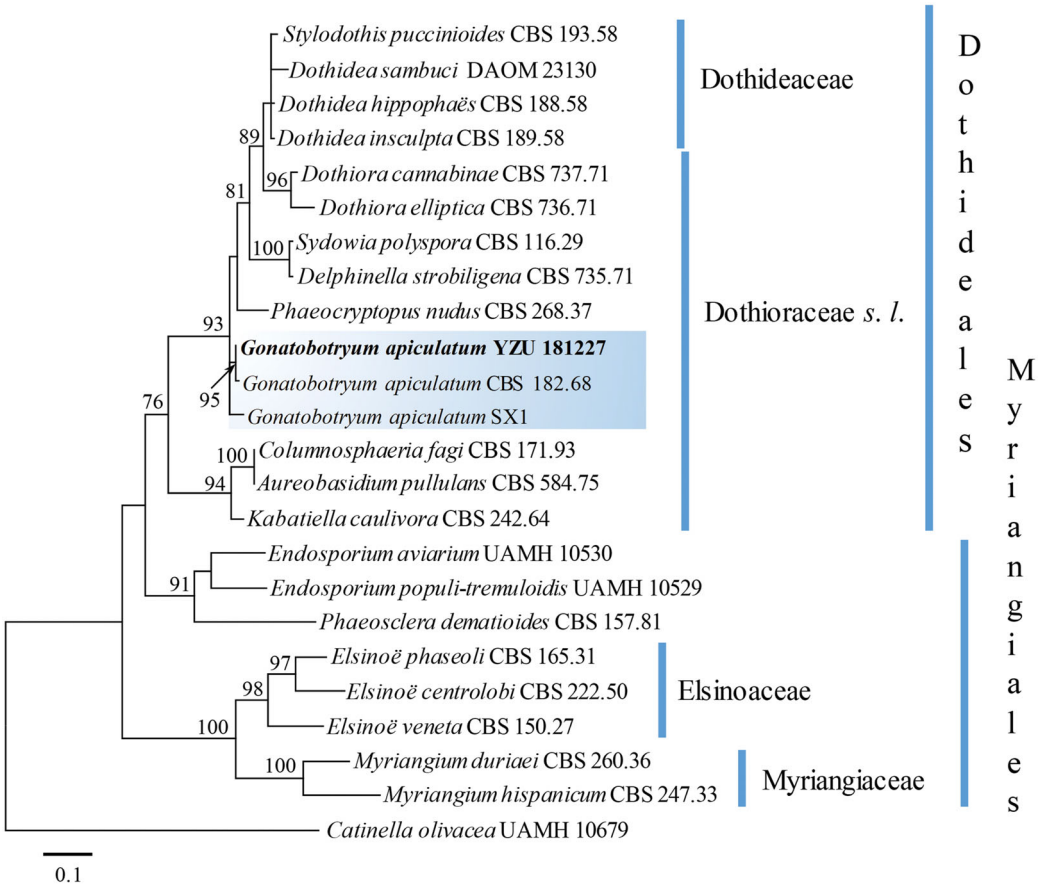

Figure 2. Phylogenetic analysis of *Gonatobotryum apiculatum* based on internal transcribed spacer (ITS) rDNA region using a maximum likelihood method. Numbers at nodes represent the percentage of bootstrap based on 1000 replicates. *Catinella olivacea* is used as an out group. The present isolate is marked in bold.

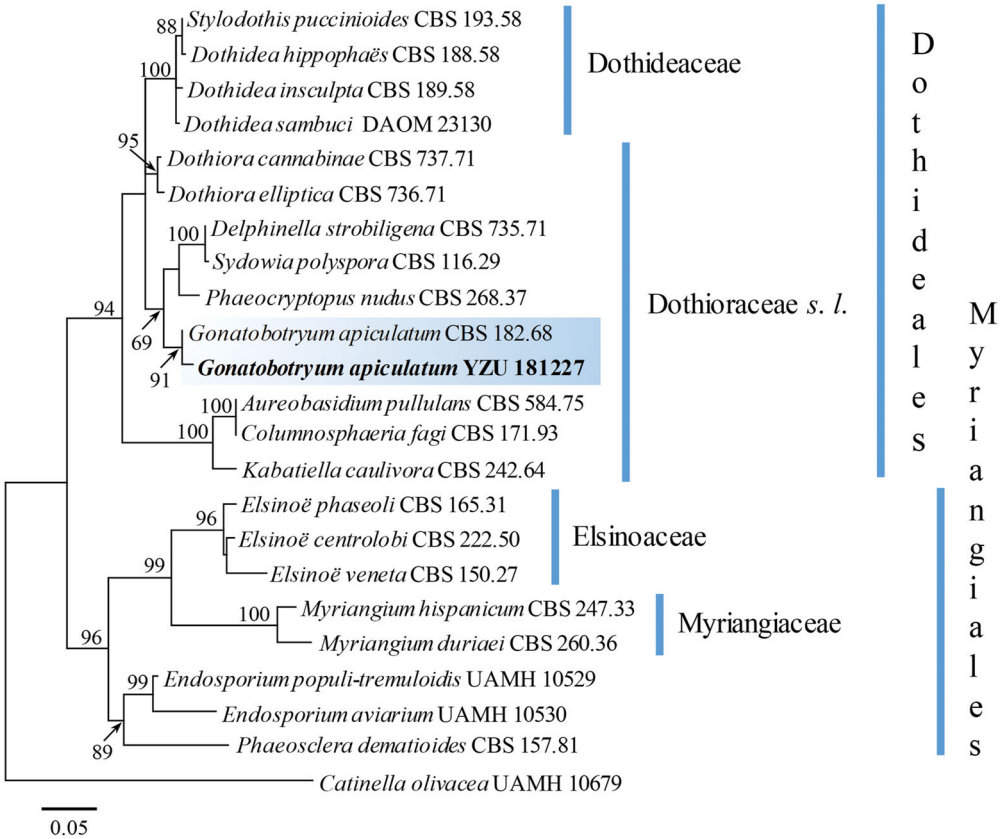

Figure 3. Phylogenetic analysis of *Gonatobotryum apiculatum* based on large subunit (LSU) rDNA region using a maximum likelihood method. Numbers at nodes represent the percentage of bootstrap based on 1000 replicates. *Catinella olivacea* is used as an out group. The present isolate is marked in bold.

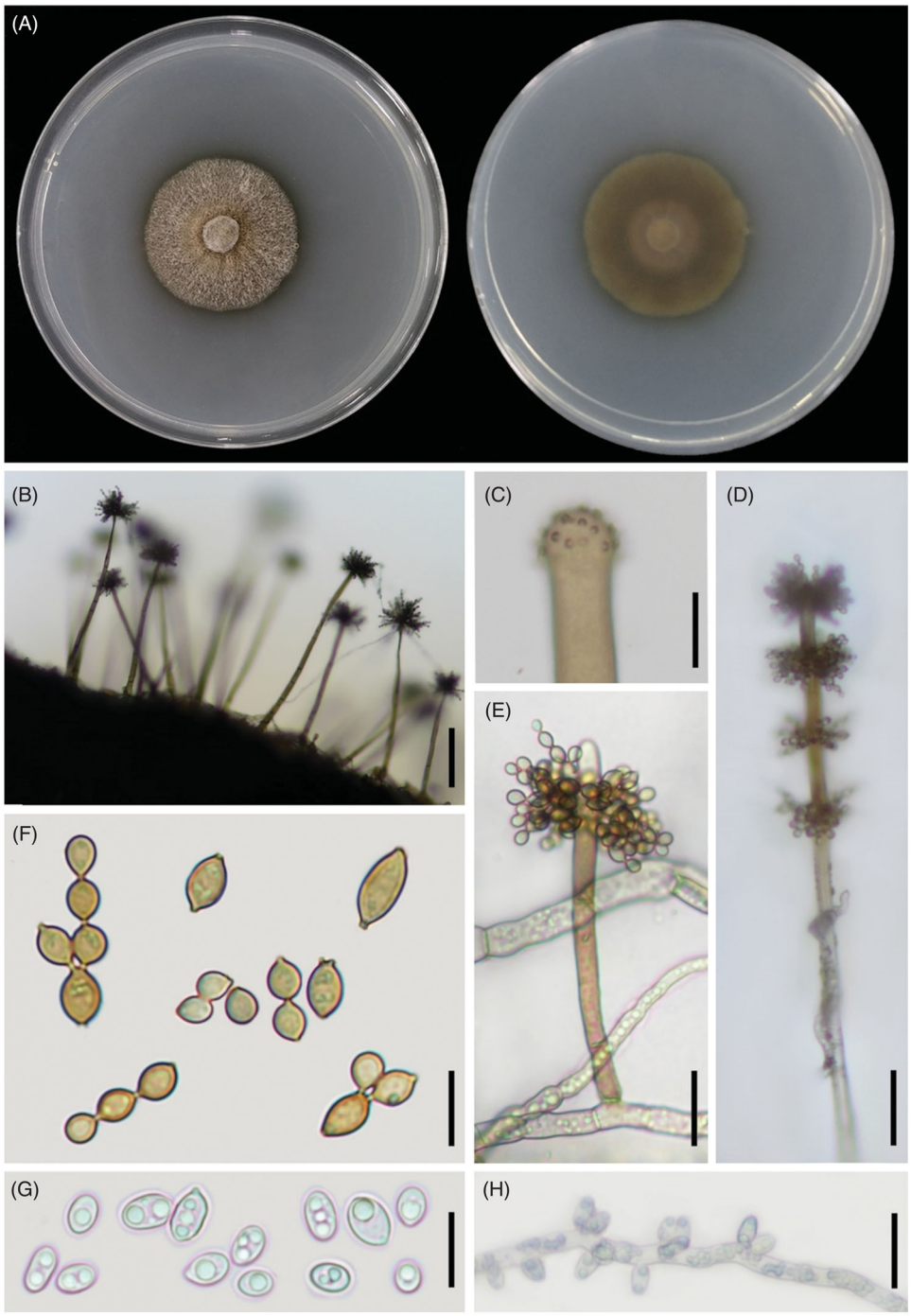

Figure 4. Morphology of *Gonatobotryum apiculatum* YZU 181227. Colony on PDA for 7 days at 25 °C (A); sporulation patterns on host (B), bar = 100 μm; a terminal conidiogenous ampullae of conidiophores (C), bar = 10 μm; conidia from conidiophores on PDA (D–F) bars: D = 40 μm, E = 20 μm, F = 10 μm; conidia on the base of colony on PDA (G) and on WA (H), bars: G = 10 μm, H = 20 μm.

leaves were surface disinfected by spraying 70% ethanol and washed with sterile distilled water for three times. Mycelia plugs were taken from 3-day-old colony grown on PDA and the spore suspension (1×10^6 spores/mL) was obtained by water flushing colonies grown on PDA. Both of them were inoculated on needle-wounded and unwounded leaves. Control groups were inoculated with pure PDA disks and distilled water. All the treated leaves were covered with clean plastic bags. Small brown necrotic spots were observed on the wounded leaves inoculated with mycelia plugs and spore suspension after two days. The lesions enlarged and reached to 10–15 mm diam after 7 days. Unwounded leaves and control groups were symptomless (Figure 1(B,C)). Diseased tissues were cultured using the same method as previous descriptions. The same fungus was re-isolated and confirmed as *Gonatobotryum apiculatum* based on conidial morphology, fulfilling the Koch's postulates. The

experiment was repeated for two times. The results indicated that *G. apiculatum* was the causal agent of leaf spot and blight on *S. henryi*.

The genus *Gonatobotryum* Saccardo (*G. fuscum* as type species) was revised by Walker and Minte based on morphological examinations with type specimens and six taxa were excluded from the genus [18]. So far, five species including *G. apiculatum*, *G. bimorphosporum*, *G. fuscum*, *G. parasiticum* and *G. piceae*, were accepted in *Gonatobotryum*. Among them, *G. fuscum* was recorded from bark and wood of various trees or as a parasite of a variety of fungi [18]. *G. parasiticum* is also reported as parasite on fungi [18]. *G. bimorphosporum* was isolated as an endophytic fungus in leaves of *Carissa carandas* [17]. *G. piceae* was found in plantula fossile Piceae sp. [19]. *G. apiculatum* has been reported from wilt leaves as a new record species in China [20], also from *Anacardium*, *Rhus*, oil of *Pinus* and as a pathogen of leaf spot on *Hamamelis* sp. [18,21]. In this study, *G. apiculatum* was the causal agent of leaf spot and blight on *Sinowilsonia henryi*. Both *Hamamelis* sp. and *Sinowilsonia henryi* were the members of Hamamelidaceae. This fungus may be a potential pathogen of plants in the family Hamamelidaceae.

In conclusion, *Gonatobotryum apiculatum* causing leaf spot and blight on *Sinowilsonia henryi* was firstly reported in China. Its morphological characteristics were determined and described in details. Phylogenetically, the species should be considered belonging to Dothioraceae sensu lato, Dothideales, Dothideomycetes, Ascomycota.

Disclosure statement

No potential conflict of interest was reported by the authors.

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