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First Report of *Botryosphaeria parva* Causing Stem Blight on *Rubus crataegifolius* in Korea

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In 2015, stem blight of *Rubus crataegifolius* was observed in Pohang, Korea. The symptoms began as dark red spots in the stem, which led to stem blight, then leaf blight, and eventually resulted in death. A fungal isolate was obtained from a symptomatic stem and incubated on a potato dextrose agar plate. The isolated fungus produced white, cloudy mycelia turned black in 3 days. Based on the morphological characteristics, the causal fungus was assumed to be *Botryosphaeria* sp. A pathogenicity test was conducted according to Koch's postulates. To identify the causal agent, the combined sequence of the internal transcribed spacer, β-tubulin, and translation elongation factor 1α genes were used for phylogenetic analysis. Approximately 1,200 bp of the combined sequence clearly suggested that the isolated pathogen was *Botryosphaeria parva*. This is the first report on stem blight in *R. crataegifolius* caused by *B. parva* in Korea.

Keywords: *Botryosphaeria parva*, Combined sequence, *Rubus crataegifolius*, Stem blight

Rubus crataegifolius Bunge belongs to the family *Rosaceae*, which is commonly found in the mountainous areas of Korea, Japan, and northeast China. *R. crataegifolius* has been used as food and traditional medicine for many years in this region by its various bioactivities such as immunological and antioxidant activities (Moon et al., 2011; Ni et al., 2009; Zhang et al., 2010). According to the usefulness of *R. crataegifolius*, the importance of disease management of *R. crataegifolius* has increased in Korea (Korea Forest Service, 2015). Several pathogens that infect *R. crataegifolius* have been reported in Korea, including *Septoria rubi*, *Rhizopus stolonifer*, *Phragmidium griseum*, and *Phytoplasma* (Kim et al., 2009). In July 2015, stem blight symptoms were observed in a field of *R. crataegifolius* in Pohang, Gyeongbuk province, Korea. Early in the course of the disease, plants showed dark red spots on stems; these spots expanded to black lesions associated with stem blight (Fig. 1A). As the disease progressed, pycnidia appeared on the stem lesions (Fig. 1B). The blight eventually spread to the leaves and resulted in plant death.

A fungus, the presumed causal agent, was isolated from the diseased plant and designated as PPL02. The infected part of the stem was cut into small pieces and the surface of pieces was sterilized with 1% NaOCl for 1 minute and washed three times with 70% ethanol. The surface-sterilized pieces were placed onto potato dextrose agar (PDA) medium and incubated at 25°C. For isolation of a pure culture, hyphal tips of mycelia on the PDA medium were cut and transferred to fresh PDA plates.

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Fig. 1. (A) Symptoms of stem blight on *Rubus crataegifolius*. (B) Conidia discharged from pycnidia on the lesion (yellow arrows). (C) Seven-day-old fungal isolate on potato dextrose agar medium. (D) Conidia. Scale bar=10 μm. (E) Symptoms following experimental inoculation.

The isolated pathogen PPL02 produced white, cloudy mycelia that reached the edge of the PDA plate within 7 days (Fig. 1C). The center of the colony turned black after 3 days of incubation, with several dark spots appearing on the back side of the plate, and the black part extended all over the plate within 21 days of incubation. When the fungal colony was exposed to visible light with a 12-hour photoperiod for 21 days, the fungus produced conidia, which were aseptate, hyaline, and ellipsoid, ranging from 13–18 μm×4–6 μm (Fig. 1D). The morphology of the colonies and conidia showed similar characteristics to *Botryosphaeria* species. Although the culture characteristics and morphologies of *B. parva* and *B. dothidea* are similar, guidelines to distinguish these species have been previously suggested (Phillips, 2002). According to the previous report, conidia of *B. parva* have 14 to 18 μm length with length to width (L/W) ratio 3.2 to 3.9 while conidia of *B. dothidea* have 20 to 28 μm length with L/W ratio 4.3 to 5.2 (Phillips, 2002). Previously reported *B. parva* isolates from various hosts have satisfied these criteria (Haleem et al., 2012; Linaldeddu et al., 2007; Phillips, 2002; Slippers et al., 2005). Thus, morphological and cultural observation suggested that PPL02 was presumed to be *B. parva*, based on its morphological and culture characteristics (Table 1).

To verify pathogenicity of PPL02, a fungal disc was obtained from the PDA plate at 7 days of incubation. The fungal disc was attached to an artificially wounded stem of a healthy plant of mature *R. crataegifolius* and then was wrapped with parafilm to maintain humidity. Ten days after inoculation, the same symptoms that were observed in the original plant appeared on the inoculated stem (Fig. 1E). Re-isolated pathogen from the disease lesion on the inoculated stem exhibited the same morphological characteristics of the original isolate. Therefore, PPL02 fulfilled Koch's postulates for establishing the causative agent of blight on *R. crataegifolius*.

Table 1. Morphological comparision of the isolate obtained from *Rubus crataegifolius* Bunge and of *Botryosphaeria* species described previously

		Present isolate	*B. parva**	*B. dothidea**
Colony color		White to black	White to black	White to black
Conidia	Size (μm)	13–18×4–6	14–19×5–7	19–27×4–6
	Setae	Absent	Absent	Absent
	Shape	Ellipsoid	Ellipsoid	Ellipsoid, fusiform
	L/W	3.4	3.3	4.1

L/W, the ratio of length to width of conidia.
*Described by Pennycook and Samuels (1985) (*Mycotaxon* 24: 445-458).

A phylogenetic analysis was performed to clarify the species of this causal agent of stem blight in *R. crataegifolius*. Genomic DNA was extracted from PPL02, grown on the PDA plate, using a Higene™ Genomic DNA Prep Kit (Biofact, Daejeon, Korea). PCR was performed with genomic DNA to amplify the internal

transcribed spacer (ITS) region, β-tubulin, and translation elongation factor 1α (EF1-α) genes. Since some species belonging to *Botryosphaeria* genus are indistinguishable in the phylogenetic analysis using ITS only (Slippers et al., 2004), in this study, we used three genes and its combined sequences for further analysis. Each gene was amplified with the following, respective primer sets: ITS1F/ITS4 for ITS region (White et al., 1990), Bt2a/Bt2B for β-tubulin (Glass and Donaldson, 1995), and EF1-728F/EF1-986R for EF1-α (Carbone and Kohn, 1999). The sequence of each gene was compared with sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) by BLAST search. Nucleotide sequences of each gene showed >99% similarity with sequences of *B. parva* (accession Nos. EU938334, FJ238525, DQ487158). Analyzed sequence data were deposited

Table 2. Isolates of *Botryosphaeria* and *Guignardia* species considered in this study

Isolates	Identity	Host	Accession No.		
			ITS	β-Tubulin	EF1-α
CMW991	*Botryosphaeria dothidea*	*Populus nigra*	AF241175	AY236924	AY236895
CMW7780	*B. dothidea*	*Fraxinus excelsior*	AY236947	AY236925	AY236896
CMW7999	*B. dothidea*	*Ostrya* sp.	AY236948	AY236926	AY236897
CMW8000	*B. dothidea*	*Prunus* sp.	AY236949	AY236927	AY236898
CMW9075	*B. dothidea*	*P. nigra*	AY236950	AY236928	AY236896
CMW9078	*B. parva*	*Actinidia deliciosa*	AY236940	AY236914	AY236885
CMW9079	*B. parva*	*A. deliciosa*	AY236941	AY236915	AY236886
CMW9080	*B. parva*	*P. nigra*	AY236942	AY236916	AY236887
CMW9081	*B. parva*	*P. nigra*	AY236943	AY236917	AY236888
CMW1130	*B. parva*	*Sequoia gigantea*	AY236945	AY236919	AY236890
CMW7054	*B. ribis*	*Ribes rubrum*	AF241177	AY236908	AY236879
CMW7772	*B. ribis*	*Ribes* sp.	AY236935	AY236906	AY236877
CMW7773	*B. ribis*	*Ribes* sp.	AY236936	AY236907	AY236878
CMW992	*B. lutea*	*A. deliciosa*	AF027745	AY236923	AY236894
CMW9076	*B. lutea*	*Malus domestica*	AY236946	AY236922	AY236893
CMW10309	*B. lutea*	*Vitis* sp.	AY339258	AY339266	AY339250
CMW6837	*B. australis*	*Acacia* sp.	AY339262	AY339254	AY339270
CMW9072	*B. australis*	*Acacia* sp.	AY339260	AY339252	AY339268
CMW9073	*B. australis*	*Acacia* sp.	AY339261	AY339253	AY339269
CMW10126	*B. eucalyptorum*	*Eucalyptus grandis*	AF283687	AY236921	AY236892
CMW6233	*B. eucalyptorum*	*Eucalyptus nitens*	AY615138	AY615122	AY615130
CMW10125	*B. eucalyptorum*	*E. grandis*	AF283686	AY236920	AY236891
CMW7774	*B. obtusa*	*Ribes* sp.	AY236953	AY236931	AY236902
CMW7775	*B. obtusa*	*Ribes* sp.	AY236954	AY236932	AY236903
CMW7060	*B. stevensii*	*F. excelsior*	AY236955	AY236933	AY236904
UCP130	*B. stevensii*	*Citrus* sp.	JF271751	JF271769	JF271787
CMW9074	*B. rhodina*	*Pinus* sp.	AY236952	AY236936	AY236901
CMW10130	*B. rhodina*	*Vitex donniana*	AY236951	AY236929	AY236900
CMW7063	*Guignardia philoprina*	*Taxus baccata*	AY236956	AY236934	AY236905
PPL02*	*B. parva*	*Rubus cratargifolius*	LC120826	LC120827	LC120828

ITS, internal transcribed spacer; EF1-α, elongation factor 1α.
*The sequence obtained in this study.

in GenBank (accession Nos. LC120826 for ITS region, LC120827 for β-tubulin, and LC120828 for EF1-α). For the phylogenetic analysis, reference genes of various species belonging to the genus *Botryosphaeria* were obtained from GenBank (Table 2). Sequences of three genes for each species, including the fungal isolate characterized in this study, combined as follows: ITS region, β-tubulin, and EF1-α. Combined sequences were aligned and analyzed using analysis tool provided in the DDBJ (DNA databank of Japan) homepage (http://www.ddbj.nig.ac.jp/). A phylogenetic tree was constructed using TreeView program with the combined sequences (Page, 1996). A results showed that our combined sequence was clustered with *B. parva* sequences (Fig. 2). Taken togather, the pathogen causing stem blight on *R. crataegifolius* was identified as *B. parva*, based on morphological and phylogenetic analyses.

B. parva was first reported to cause soft rot on kiwifruit in New Zealand (Pennycook and Samuels, 1985). Since this first report, presence of *B. parva* has been reported in 71 host species

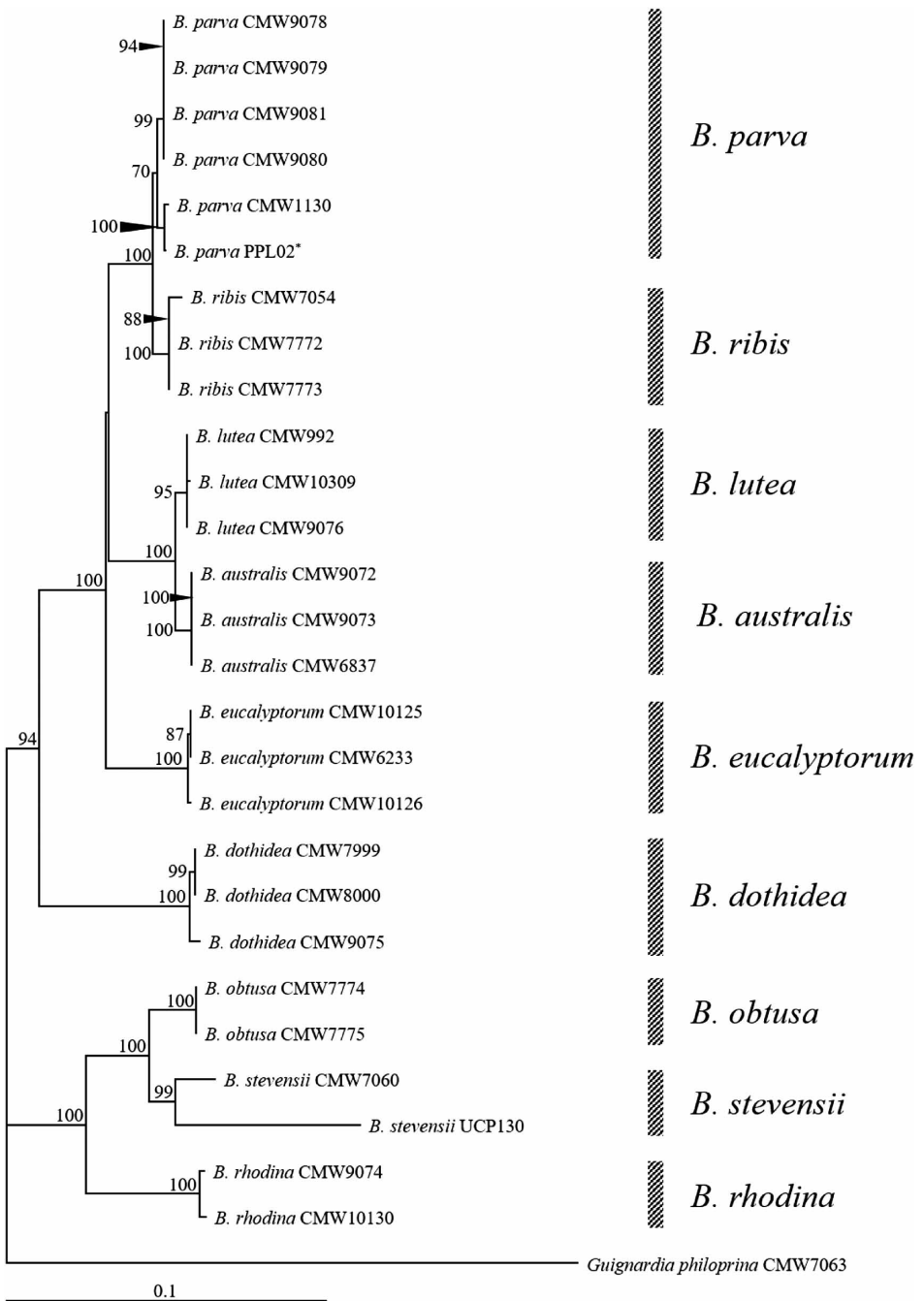

Fig. 2. Phylogenetic analysis of *Botryosphaeria* species using a combined sequence of the internal transcribed spacer, β-tubulin, and elongation factor 1α genes. The tree was constructed using the neighbor-joining method with 1,000 replicates. *The fungus isolated in this study.

across six continents and 21 countries (Sakalidis et al., 2013). This pathogen causes serious symptoms, including dieback, canker, stem blight, and fruit rot in various hosts, such as grapevines, kiwi vines, cherry trees, raspberry bushes, and mango (Abdollahzadeh et al., 2013; Haleem et al., 2012; Linaldeddu et al., 2007; Pennycook and Samuels, 1985; Phillips, 2002; Sakalidis et al., 2013; Slippers et al., 2005; van Niekerk et al., 2004). Thus, the characterization and management of this pathogen is very important. In Korea, *B. parva* was not reported until 2009 (Kim et al., 2009). In 2012 and 2013, dieback symptoms caused by *Neofusicoccum parvum*, an anamorphic synonym of *B. parva*, on blueberries and walnuts has been reported (Cheon et al., 2013; Choi et al., 2012), but there has been no report hitherto that *B. parva* (*N. parvum*) was isolated from *R. crataegifolius*. Thus, this is the first report of stem blight on *R. crataegifolius* caused by *B. parva* in Korea. Considering economic value of *R. crataegifolius*, methods to manage this pathogen are urgently needed to prevent low production of *R. crataegifolius*.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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