

## Identification and Characterization of the Causal Organism of Gummy Stem Blight in the Muskmelon (*Cucumis melo* L.)

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Gummy stem blight is a major foliar disease of muskmelon (*Cucumis melo* L.). In this study, morphological characteristics and rDNA internal transcribed spacer (ITS) sequences were analyzed to identify the causal organism of this disease. Morphological examination of the Jeonbuk isolate revealed that the percentage of monoseptal conidia ranged from 0% to 10%, and the average length × width of the conidia was 70 (± 0.96) × 32.0 (± 0.15) μm on potato dextrose agar. The BLAST analysis showed nucleotide gaps of 1/494, 2/492, and 1/478 with identities of 485/492 (98%), 492/494 (99%), 491/494 (99%), and 476/478 (99%). The similarity in sequence identity between the rDNA ITS region of the Jeonbuk isolate and other *Didymella bryoniae* from BLAST searches of GenBank was 100% and was 95.0% within the group. Nucleotide sequences of the rDNA ITS region from pure culture ranged from 98.2% to 99.8%. Phylogenetic analysis with related species of *D. bryoniae* revealed that *D. bryoniae* is a monophyletic group distinguishable from other *Didymella* spp., including *Ascochyta pinodes*, *Mycosphaerella pinodes*, *M. zaeae-maydis*, *D. pinodes*, *D. applanata*, *D. exigua*, *D. rabiei*, *D. lentis*, *D. fabae*, and *D. vitalbina*. Phylogenetic analysis, based on rDNA ITS sequence, clearly distinguished *D. bryoniae* and *Didymella* spp. from the 10 other species studied. This study identified the Jeonbuk isolate to be *D. bryoniae*.

**KEYWORDS :** *Didymella bryoniae*, Gummy stem blight, Muskmelon, rDNA ITS sequencing

The muskmelon (*Cucumis melo* L.) is a member of family Cucurbitaceae and a native of East Africa and is currently cultivated worldwide. In Korea, 45 pathogenic species have been reported in muskmelon. No information available which indicates that *Didymella bryoniae* causes gummy stem blight in muskmelon; however, some data indicate that it causes such in watermelon [1, 2]. *D. bryoniae* has a broad host range, causing conditions viz. black rot, gummy stem blight, leaf spot, and stem canker [3-5]. *D. bryoniae* is ascomycetes, which causes infections in stems by pycnosporo- and perithecia-containing ascospores. Most infections occur during the rainy season, when the humidity is ≥ 90% and the temperature is 20–24°C [5]. During the winter, *D. bryoniae* hibernates and can survive up to 5 mon on the soil surface [6]. Infected stems manifest irregular-shaped grayish-brown spots and ooze reddish mucus early in the infection. Over time, the mucus dries on the stem and forms many small black spots (pycnidia). Large irregular-shaped spots appear on the leaves of the plant, whereas the stalk of the fruit rots and turns brown in color [5]. *In vitro*, *D. bryoniae* does not produce pycnidium in the absence of irradiation, but pycnidiospores produce mycelium very quickly if cultured under ultraviolet (UV) light and in the dark [3]. It has also been reported that UV light is required for the sporulation of *D. bryoniae*. In a greenhouse, the onset of

disease can be controlled by using UV-absorbing vinyl film [7].

Gummy stem blight is the most destructive foliar disease of cucurbits. A method for the rapid diagnosis and analysis of genetic variability in *D. bryoniae* has proven very useful [8, 9]. A comparison of DNA components in the ribosomal unit, such as the 5.8S, 18S, and 28S RNA encoding genes in fungi, allows separation of organisms at the gene level. The internal transcribed spacer (ITS) is more specific region for distinction at the species level [10]. Thus, the objective of the present study was to identify the causal agent of gummy stem blight in muskmelon by conducting a morphological examination and a sequence analysis of the ITS region of rDNA.

### Materials and Methods

**Isolation and observation of morphological characteristics of the pathogen.** An earless kingstar cultivar of muskmelon (*Cucumis melo* L.) was cultivated in a greenhouse at Jeollabuk-do Agriculture Research and Extension Services (JBARES), Iksan (latitude: 35°58' N; longitude: 127°2' E), from April to August 2009. Gummy stem blight was observed on the stem and leaf stalk of muskmelon during the rainy season. No chemicals were used to treat the gummy stem blight; thus, the disease was allowed to spread naturally. Infected samples from the plants were collected to isolate and identify the pathogen.

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Skin from the diseased portion of the plant was surface-sterilized with 1% NaOCl for 1 min and was washed three times with sterile de-ionized water to remove the NaOCl. Any remaining water was wiped off with sterilized filter paper. The pathogen was isolated from the inner part of the stem and cultured on potato dextrose agar (PDA; Difco, Sparks, MD, USA) in the dark at 25°C for 3 days. The end parts of hypha cultured on PDA were transferred aseptically onto malt extract agar (Difco) and V-8 juice agar (Campbell Soup Co., Camden, NJ, USA) and incubated at 25°C in the dark for 10 days. Fruiting bodies (conidia and conidiophores) from the pathogen were surface-sterilized and placed on PDA for growth under a 12-hr photoperiod for an additional wk to induce sporulation [11]. A morphological examination was then conducted. Fruiting bodies were placed on a droplet of sterile water on a microscopic slide and crushed to release the contents. The length  $\times$  width of the conidia from the pathogen was measured at 400 $\times$  magnification and was microscopically examined for the presence of septae (Nikon 80i; Nikon, Tokyo, Japan). The size of conidia and conidiophores was measured by using NIS elements BR-300 software (Nikon). The diameter of the colonies in the isolate culture was measured at right angles. The morphological data were compared with previously collected data for *D. bryoniae*.

**PCR amplification of the rDNA ITS region and sequence analysis.** To obtain pure mycelium, a small amount of tissue was taken from the isolate and cultured on PDA in the dark at 25°C for 1 wk. The pure mycelium was used for a sequence analysis of the rDNA ITS region (ITS-1 region, 5.8S gene, and ITS-2 region). Genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Amplification and sequencing of the isolate (in the region containing ITS1, ITS2, and 5.8S rDNA) was performed using a pair of universal primers - ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [9] - which were also used as a positive control in a subsequent diagnostic PCR. The amplification was carried out in a 20- $\mu$ L reaction mixture containing 50 ng of genomic DNA, 10 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% gelatin, 0.2 mM dNTP, 200 ng of each primer, and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA). The reaction mixtures were denatured at 94°C for 10 min and subjected to 35 cycles of 1 min each at 94°C, annealing at 56–60°C for 1 min, extension at 72°C for 1 min, and a final extension step of 7 min at 72°C. An amplified PCR product was separated on 1.5% agarose gel and purified with a DNA purification kit (Core-one™; Core-bio, Seoul, Korea) according to the manufacturer's instructions. Both amplicon strands were sequenced using the same primers

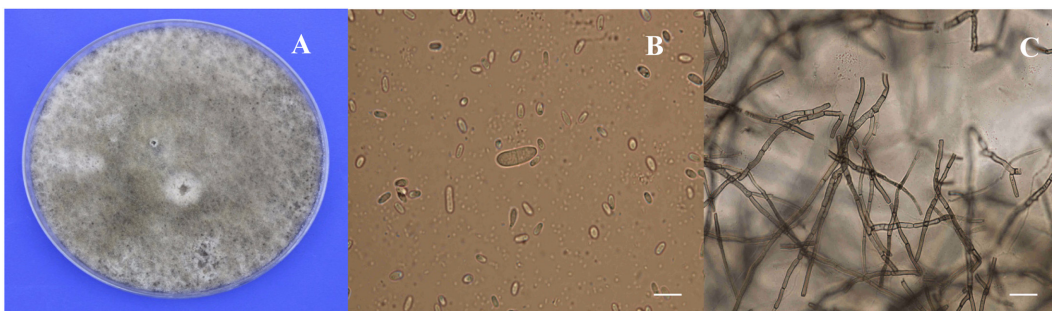
used for the initial amplification. The reactions were monitored using BigDye terminator cycle sequencing kits (Applied Biosystems, Foster City, CA, USA) as indicated by the manufacturer and run on an ABIPRISM 3130 automated DNA sequencer (Applied Biosystems) as described previously [12]. The rDNA ITS sequence data were analyzed using the DNASTAR program (DNASTAR Inc., Madison, WI, USA) and aligned by using the CLUSTAL W method [13]. Sequence data from the ribosomal ITS genes of *Didymella* spp. were used to perform a preliminary phylogenetic analysis; 16 ITS sequences were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov/>) (Table 2). Phylogenetic analysis based on parsimony for ITS, was obtained from the data by using neighbor-joining methods with MEGA ver. 4.0 software [14, 15], and the sequence distance was calculated using the Tamura-Nei, parameter model. Bootstrap analysis was performed with 1,000 replications to determine the support for each clade [16]. The MegAlign program (DNASTAR Inc.) was used to compare the percentage sequence identity from the Jeonbuk isolate with that of other *Didymella* spp. in GenBank [17].

## Results and Discussion

**Symptoms of disease and its morphological characteristics.** *D. bryoniae* spreads during the rainy season (July to August). In early July, less than 5% of the plants examined were infected (15 of 300 plants); however, in early August, 18.4% of the plants were infected. Gummy stem blight initially attacks the main stem and then spreads to the branches of the muskmelon. Disease symptoms include stem necrosis, gummy exudation, angular water-soaked lesions on the leaves, and rotten fruit. The weight of the sticky grayish-brown mucus that oozes from the infected part of the plant eventually causes the branches of the plant to break down (Fig. 1A and 1C). After some time, grayish-brown mucus turns dark brown in color (Fig. 1B). Black pycnidia were observed on the stems, leaves, and fruit of the muskmelon. A preliminary identification of the fungi was based on the content of conidia and conidiophores, as described in the Material and Methods section. The mycelia of *D. bryoniae* from the PDA culture were white to dark gray on top and black on the bottom. After 15 days, the *D. bryoniae* produced a conidial mass with a white aerial mycelium at the center of colony; few pycnidia were observed. After 10 days, the *D. bryoniae* colonies on the malt extract agar and V-8 juice agar varied in diameter from 8.5 to 10.5 cm and extended to the edge of the petri dish (Fig. 2A). The percentage of monosepta in the conidia of *D. bryoniae* ranged from 0% to 10%, and the mean length  $\times$  width of the conidia in the PDA was 70 ( $\pm$ 0.96)  $\times$  32.0 ( $\pm$ 0.15)  $\mu$ m (Fig. 2B and 2C). Our Jeonbuk isolate had morpho-



**Fig. 1.** Symptoms of gummy stem blight on muskmelon plants caused by *Didymella bryoniae*. A and B, Infected stem in a mature melon; C, Broken stem at an early stage.



**Fig. 2.** Culture and morphological characteristics of the pathogenic fungi *Didymella bryoniae*. A, Colony formed on potato dextrose agar after 10 days of incubation. Note the white conidial mass in the center of the colony. B and C, Conidia and mycelia of *D. bryoniae* cultured on potato dextrose agar after exposure to ultraviolet light for 12 hr (scale bar = 25  $\mu$ m).

logical characteristics similar to those reported by Keinath *et al.* [11], Lee [4], and Somai *et al.* [18]. In agreement with previous reports, the cultures of *D. bryoniae* grown on PDA produced olive to dark-green or black substrate mycelium and white and hairy aerial mycelium. Compared with growth on PDA and malt extract agar, growth on V-8 juice agar tended to occur more frequently in concentric zones for both groups. Characteristics such as the size of the conidia and the percentage of septa have been used to study the taxonomy of the genus *D. bryoniae* and *Phoma* spp. [11]. The size of the conidia and the percentage of monosepta in *D. bryoniae* were about the same as described previously. Some reports suggest that the presence of septae may not be a useful characteristic by which

to distinguish the anamorph of *D. bryoniae* from *Phoma* spp. Therefore, additional morphological characteristics and more sensitive methods are needed to differentiate between these closely related species.

**PCR amplification of the rDNA ITS region and sequence analysis.** Total 17 rDNA ITS sequence data for *Didymella* spp. were compared and analyzed for similarities. A phylogenetic analysis of the Jeonbuk isolate, which was isolated from the field of JBABRES, eight species of *Didymella* spp., two species of *Ascochyta* spp., and two species of *Mycosphaerella* spp. all registered as part of the genus *Didymella* in GenBank was conducted.

The size of the entire rDNA ITS region, which was

**Table 1.** Comparison of nucleotide sequences in the rDNA internal transcribed spacer region of Jeonbuk isolate with those of other *Didymella bryoniae* from BLAST searches of GenBank

Identified name by BLAST	NCBI accession no.	Analysis sequence (bp)	Nucleotide gaps (%)	Nucleotide identity (%)	Match score (bit) <sup>a</sup>	E-value <sup>b</sup>
<i>D. bryoniae</i> I	EF160074	515	1/494	492/494 (99)	879 (974)	0
<i>D. bryoniae</i> II	EF160076	515	1/494	491/494 (99)	874 (968)	0
<i>D. bryoniae</i> III	EF107641	515	2/492	485/492 (98)	850 (942)	0
<i>D. bryoniae</i> IV	EF107642	515	1/494	492/494 (99)	879 (974)	0
<i>D. bryoniae</i> V	AF297228	553	1/478	476/478 (99)	850 (942)	0

<sup>a</sup>Match score was calculated by nucleotide that agree to the given score.

<sup>b</sup>E-value indicates the number of hits expected to occur by chance with the given score.

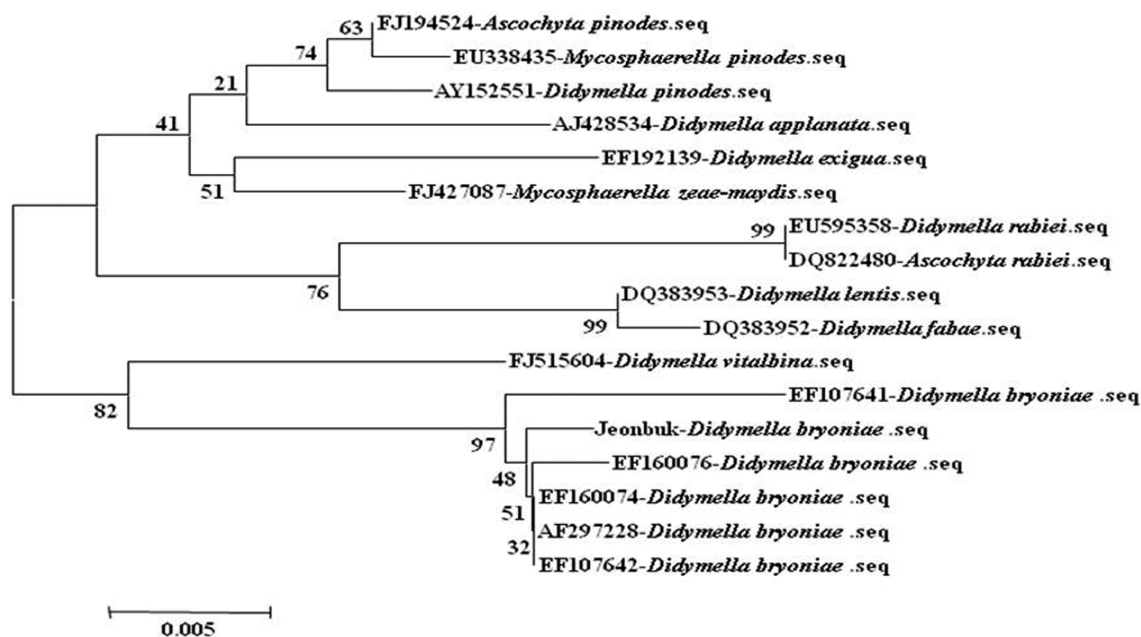
**Table 2.** Comparison of percentage of sequence identity in the rDNA internal transcribed spacer region of Jeonbuk isolate with that of other *Didymella bryoniae* grouped in GenBank

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		96.6	97.5	96.8	97.3	96.8	96.6	97.5	97.0	97.3	99.8	100.0	98.4	100.0	96.6	96.6	99.8
2			96.8	96.1	96.6	96.8	100.0	96.6	96.6	96.8	96.1	96.3	95.4	96.3	97.7	97.7	96.1
3				98.4	99.8	98.6	96.8	97.3	99.3	98.6	97.3	97.5	95.9	97.5	97.3	97.3	97.7
4					98.2	97.5	96.1	97.0	97.7	98.4	96.6	96.8	95.2	96.8	96.1	96.1	97.0
5						98.4	96.6	97.0	99.1	98.4	97.0	97.3	95.7	97.3	97.3	97.3	97.5
6							96.8	96.8	98.4	98.6	96.8	96.8	95.2	96.8	97.7	97.7	97.0
7								96.6	96.6	96.8	96.1	96.3	95.4	96.3	97.7	97.7	96.1
8									97.0	97.7	97.3	97.5	96.1	97.5	96.1	96.3	97.3
9										98.4	96.8	97.0	95.4	97.0	97.0	97.0	97.3
10											97.3	97.3	95.7	97.3	96.8	96.8	97.5
11												99.8	98.2	99.8	96.4	96.3	99.5
12													98.4	100.0	96.6	96.6	99.8
13														98.4	95.0	95.0	98.2
14															96.6	96.6	99.8
15																99.8	96.1
16																	96.1
17																	

1, AF297228; 2, EU595358; 3, FJ194524; 4, EF192139; 5, EU338435; 6, AJ428534; 7, DQ822480; 8, FJ515604; 9, AY152551; 10, FJ427087; 11, EF160076; 12, EF160074; 13, EF107641; 14, EF107642; 15, DQ383953; 16, DQ383952; 17, *D. bryoniae*-Jeonbuk.

amplified using primers ITS1 and ITS4 from a *D. bryoniae* isolate, was 500 bp and yielded 440 aligned nucleotide positions for all species included in the alignment. The nucleotide sequences in the rDNA ITS region of the Jeonbuk isolate were compared with those of the other *D. bryoniae* spp. (Table 1) in a BLAST search of the GenBank database. The BLAST analysis showed nucleotide gaps of 1/494, 2/492, and 1/478 with identities of 485/492 (98%), 492/494 (99%), 491/494 (99%), and 476/478 (99%).

The Jeonbuk isolate was identified as *D. bryoniae*. Data from the entire rDNA ITS sequence was analyzed using the DNASTAR program and was aligned using the CLUSTAL W mode (Table 2). The similarity in sequence identity between the rDNA ITS region of the Jeonbuk isolate and other *D. bryoniae* from BLAST searches of GenBank was 100% and was 95.0% within the group. The nucleotide sequences in the rDNA ITS region, obtained from pure cultures of the Jeonbuk isolate, were 98.2~

**Fig. 3.** Phylogenetic tree for the rDNA internal transcribed spacer sequence from *Didymella bryoniae* and for reference sequences obtained from GenBank (by Laser gene 7.0).

99.8% similar to those of isolates with the accession numbers EF160074, EF160076, EF107641, EF107642, and AF297228 in GenBank, which were similar to the deposited sequences from the *D. bryoniae* isolates (Table 2). The phylogenetic analysis of species related to *D. bryoniae* revealed that *D. bryoniae* is a monophyletic group distinguishable from other *Didymella* spp., including *Ascochyta pinodes*, *Mycosphaerella pinodes*, *M. zaeae-maydis*, *D. pinodes*, *D. applanata*, *D. exigua*, *D. rabiei*, *D. lentis*, *D. fabae*, and *D. vitalbina*.

The phylogenetic analysis based on rDNA ITS sequences clearly distinguished *D. bryoniae* and *Didymella* spp. Of the other 10 species, the *D. bryoniae* group had a bootstrap value of 97%, whereas the remaining species had a bootstrap value of 21–99% (Fig. 3). In the current study, the Jeonbuk isolate was identified as *D. bryoniae* on the basis of morphological characteristics and a comparison of rDNA ITS sequence data with other related data from GenBank and previous studies. The nucleotide sequences in the rDNA ITS region of the Jeonbuk isolate were 98.2–99.8% similar to the sequences of *D. bryoniae* isolates in GenBank. The phylogenetic analysis showed that *D. bryoniae* is a monophyletic group distinguishable from other *Didymella* spp. Somai *et al.* [18] conducted random amplified polymorphic DNA (RAPD) and ITS sequence analyses to distinguish *D. bryoniae* from *Phoma* spp. isolated from cucurbits. Keinath *et al.* [11] successfully distinguish *D. bryoniae* from *Phoma* spp. on the basis of morphological characteristics and PCR-based RAPD analysis. Shim *et al.* [2] also analyzed the genetic diversity of *D. bryoniae* on the basis of RAPD profiles, which substantiated by sequence characterized amplified regions marker in Korea. These results indicated that the genus *D. bryoniae* was not monophyletic, and *D. bryoniae*, *D. pinodes*, *D. rabiei*, *D. lentis*, and *D. vitalbina* were distinct based on a sequence analysis of ITS1, ITS2, and 5.8S rDNA. Additional studies are needed to develop biological and eco-friendly methods to control gummy stem blight in muskmelon to prevent damage caused by *D. bryoniae*.

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