

Population Structure of *Stagonosporopsis* Species Associated with Cucurbit Gummy Stem Blight in Korea

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Gummy stem blight (GSB), a common and serious disease in cucurbits worldwide, is caused by three genetically distinct species: *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*), *S. citrulli*, and *S. caricae*. In Korea, however, the three species of *Stagonosporopsis* have been barely characterized. In this study, 21 *Stagonosporopsis* isolates were recovered from watermelon (*Citrullus lanatus*) and muskmelon (*Cucumis melo*) leaves and stem showing blight symptoms collected from 43 fields in Korea. Sequence analysis performed with an internal transcribed spacer region was not competent to differentiate the *Stagonosporopsis* isolates. On the contrary, analysis of β -tubulin (*TUB*) genes and three microsatellite markers, Db01, Db05, and Db06, successfully differentiated *Stagonosporopsis* isolates. Further sequence analysis identified two *Stagonosporopsis* species, *S. citrulli* and *S. caricae*, and one previously unknown species of *Stagonosporopsis*. Representative isolates from three species caused dark water-soaked lesions on the detached watermelon and muskmelon leaves with no significant differences in the aggressiveness. Our results indicate that the *S. citrulli*, *S. caricae*, and unknown *Stagonosporopsis* sp. are all caus-

al agents of GSB for both watermelon and muskmelon. This is the first report of a new species and the population structure of *Stagonosporopsis* species causing GSB in Korea.

Keywords : cucurbits, gummy stem blight, *Stagonosporopsis*

Watermelon (*Citrullus lanatus* Thunb.) and muskmelon (*Cucumis melo* L.), members of the Cucurbitaceae family (cucurbits), are economically important fruit crops, and their annual production in 2019 was estimated more than 120 million tons worldwide (Food and Agriculture Organization of the United Nations, 2019). It is widely grown in the most parts of Africa, the Southern United States, and Southeast Asia (Ma et al., 2021).

Gummy stem blight (GSB), also known as black rot when affecting fruits, is a devastating disease and a major production constraint of the Cucurbitaceae family (Wolukau et al., 2007). This disease was first observed on *Cucumis sativus* (cucumber) in France and on watermelon in the United States in 1892 (Gusmini et al., 2017) and now assumed to be distributed worldwide. Disease symptoms include foliar brown discoloration and necrotic lesions on the infected stems and fruits (Basim et al., 2016; Li et al., 2015). Fungicide applications are the main method to control GSB on cucurbits (Keinath, 2021). Therefore, identification of causal agents of the GSB is essential for choosing effective chemicals and setting up disease-management programs.

Didymella bryoniae was initially reported as a fungal pathogen causing the GSB on cucurbits worldwide including China, the United States, and Korea (Jensen et al.,

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2011; Koike, 1997; Kwon et al., 1997; Ling et al., 2010; Sudisha et al., 2004). Later, *Stagonosporopsis cucurbitacearum* was described as a synonym of *D. bryoniae* causing GSB (Aveskamp et al., 2010). However, a multi-locus sequencing study of diverse isolates of *Stagonosporopsis* species showed that GSB is caused by three morphologically similar, but genetically distinct *Stagonosporopsis* spp., *S. cucurbitacearum*, *S. citrulli*, and *S. caricae* (Brewer et al., 2015; Stewart et al., 2015). During the reclassification of the *Stagonosporopsis* spp., some of phylogenetically distinct isolates, previously classified as *S. cucurbitacearum*, were reassigned to the new species, *S. citrulli* (Stewart et al., 2015). One of the species, *S. caricae*, known to affect only papaya (*Carica papaya*) (Boerema et al., 2004; Brewer et al., 2015) was reported to be also aggressive on cucurbits, displaying a broader host range, and became a causal agent of GSB (Stewart et al., 2015). Previous population studies have shown that they vary in their geographic distribution worldwide (Garampalli et al., 2016; Stewart et al., 2015). *S. citrulli* was the most abundant species with the widest geographic distribution, and *S. caricae* was also widely distributed. However, *S. cucurbitacearum* has been only found in temperate regions (Garampalli et al., 2016; Stewart et al., 2015). To consistently compare GSB populations, 18 polymorphic microsatellite markers were developed, and three of them—Db01, Db05, and Db06—were successfully applied for polymerase chain reaction (PCR)-based markers for distinguishing *Stagonosporopsis* spp. (Brewer et al., 2015).

Causal agents of GSB have been commonly misidentified. For example, because *S. caricae* was described as infecting only papaya (Boerema et al., 2004; Brewer et al., 2015), some isolates of *S. caricae* infecting cucurbits had been misidentified as *S. cucurbitacearum* (Brewer et al., 2015). The host species of origin and similar morphology make many isolates of *S. citrulli* also misidentified as *S. cucurbitacearum*. Therefore, isolates previously identified as *S. cucurbitacearum* should be reidentified on the basis of its phylogenetic diversity. In addition, population structure of the GSB pathogens and diversity within and among the populations at the field level need to be determined to better understand the *Stagonosporopsis* spp. and set up GSB management strategies.

To our knowledge, there are only a few studies carrying out a molecular identification of *Stagonosporopsis* spp. causing GSB worldwide, and very few has been reported in Korea. Here, we describe the population structure of *Stagonosporopsis* spp. including one previously unknown species associated with GSB in Korea.

Materials and Methods

Plant sample collection and isolation of *Stagonosporopsis* species. From 2019 to 2022, a total of 43 fields from 14 cities among the watermelon and muskmelon production area in Korea were selected for the survey of causal agents for GSB. Leaves and stems of watermelon and muskmelon showing GSB were harvested. Small pieces of tissues (10 × 10 mm) were removed from the margins between the healthy and symptomatic tissues and their surface were disinfected with 70% ethanol for 1 min, followed by rinsing with sterilized dH₂O three times. The pieces were then placed on potato dextrose agar (PDA) (MBCcell, Seoul, Korea) plates and incubated at 28°C in the light for 7 days. Multiple fungal colonies were obtained from each symptomatic tissue and only those with *Stagonosporopsis* morphologies were selected as isolates. The isolates were sub-cultured onto fresh PDA media for single spore purification.

***Stagonosporopsis* genomic DNA extraction.** The genomic DNAs were extracted following the protocol described previously (Chen and Ronald, 1999). For the extraction, *Stagonosporopsis* mycelia were scrapped from the surface of 7-day cultures grown on PDA and placed in a 2 ml microcentrifuge tube containing 700 µl cetyltrimethylammonium bromide (CTAB) extraction buffer (3% (w/v) CTAB, 20 mM EDTA pH 8.0, 1.42 M NaCl, 100 mM Tris-HCl pH 8.0, 2% (w/v) polyvinylpyrrolidone, and 0.2% β-mercaptoethanol). After incubating for 10 min at 65°C, adding 570 µl of chloroform:isoamyl alcohol (24:1) to the tube, and centrifuging for 10 min at 13,000 ×g, genomic DNA was precipitated with 0.7 volumes of isopropanol. After centrifuging for 10 min at 13,000 ×g, the genomic DNA pellet was washed with 70% ethanol and then dried at room temperature. The genomic DNA was dissolved in 30 µl of sterilized dH₂O and stored at –20°C.

PCR amplification. The primers for the internal transcribe spacer (ITS) region of ribosomal DNA, β-tubulin (*TUB*) gene, and three microsatellite markers (Db01, Db05, and Db06) were used for PCRs (Table 1). PCR was performed in a 20 µl reaction mixture containing 0.1 µl FIREPol DNA polymerase (0.5 units) (Solis BioDyne, Tartu, Estonia), 1 µl each primer (10 µM), 0.2 µl dNTP mix (200 µM), 2 µl 10× buffer, 1 µl template (approximately 100 ng), 4 µl 5× Q-solution, and 10.7 µl sterilized dH₂O. Amplifications were performed with an initial denaturation step at 94°C for

Table 1. Primers used in this study

DNA region	Primer name	Primer sequence (5'-3')	Reference
Universal primers			
ITS	ITS1	TCCGTAGGTGAACCTGCGG	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	
<i>TUB</i>	TUB_T1_F	TCTGGATGTTGTTGGGAATCC	O'Donnell and Cigelnik (1997)
	TUB_T22_R	ACCCGCTGAACTTAAGC	
Primers for microsatellite markers			
Db01	Db01_F	CGGTCCGGTCAACCTACTAC	Brewer et al. (2015)
	Db01_R	CACGCCAGCAAATCTCACTA	
Db05	Db05_F	TATGACGTTGGGCAAGTGAG	Brewer et al. (2015)
	Db05_R	TTTGCTGGGATGGTGTGTGA	
Db06	Db06_F	GGTGACATCTGCGTGAATG	Brewer et al. (2015)
	Db06_R	TGGTTGTTGGTTGTTTGA	

ITS, internal transcribe spacer; *TUB*, β -tubulin.

3 min, followed by 35 cycles of 15 s at 94°C, 15 s at 53°C and 40 s at 72°C for ITS region, 35 cycles of 15 s at 94°C, 15 s at 54°C and 90 s at 72°C for *TUB* gene, 35 cycles of 15 s at 94°C, 15 s at 53°C and 30 s at 72°C for three microsatellite markers (Db01, Db05, and Db06) and final extension at 72°C for 7 min. Amplified fragments were loaded on an agarose gel (0.8% w/v or 1.5% w/v) stained with RedSafe Nucleic Acid Staining Solution (20,000 \times) (Intronbio, Seongnam, Korea) and visualized under UV light.

Morphological characterization. The morphology of *Stagonosporopsis* isolates was examined as previously described (Choi et al., 2010) with minor modification. For colony type, color, and margin, *Stagonosporopsis* isolates were incubated on PDA plates in a 28°C dark condition for 5 days. For spore observation, spores were recovered from watermelon cultivar ‘Seotaja’ (Lee et al., 2016) and muskmelon cultivar ‘Earls top one’ (Hassan et al., 2018) inoculated with *Stagonosporopsis* isolates, filtrated with sterilized gauze (Dong-A Health Care, Gwangju, Korea), and precipitated by centrifuging at 6,000 $\times g$ for 1 min. Harvested spores were resuspended with 100 μ l dH₂O and examined using an inverted microscope Eclipse Ti (Nikon, Tokyo, Japan) fitted with an objective (200 \times).

Phylogenetic analysis. The PCR amplification products of ITS region, *TUB*, and microsatellite marker Db05 were purified with FavorPrep GEL/PCR Purification Kit (Favorgen, Ping-Tung, Taiwan) and sequenced by Sanger sequencing (Macrogen, Seoul, Korea). Sequence similarity searches were performed using the nucleotide BLAST at National Center for Biotechnology Information (NCBI, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). DNA sequences of ITS region,

TUB, and Db05 were aligned through ClustalW and refined manually using MEGA X (Kumar et al., 2016, 2018). Maximum likelihood based on the Jukes-Cantor model of ITS region, *TUB*, and Db05 was performed using MEGA X with 500 bootstrap replicates. Branches corresponding to partitions reproduced in less than 50%. The phylogenetic trees were viewed in MEGA X. All analyzed DNA sequences of ITS region and *TUB* from isolates were deposited in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>), and their accession numbers are listed in Table 2.

Pathogenicity tests. *Stagonosporopsis* isolates were inoculated according to the previously described detached-leaf inoculation method (Pryor and Michailides, 2002) with minor modification. Briefly, to prepare inoculum, *Stagonosporopsis* isolates were pure-cultured on PDA plates at 28°C in the dark for 7 days, and each mycelial plug was cut from the actively growing margin of the fungal colonies. Fully expanded leaves were collected from 6- to 7-week-old watermelon cultivar ‘Seotaja’ and muskmelon cultivar ‘Earls top one’ grown in a growth chamber. For the inoculation of each *Stagonosporopsis* isolate, three leaves from each cultivar were placed on papers soaked with distilled water in plastic boxes (25 \times 19 \times 5 cm [length \times width \times height]), and one mycelial plug was placed on each detached leaf. The plastic boxes were individually covered with transparent plastics to maintain 100% humidity and incubated at 25°C with a 12 h photoperiod per day for 7 days. Disease severity was calculated after each disease area was divided by whole leaf area using ImageJ. *Stagonosporopsis* isolates were re-isolated consistently from all the inoculated leaf to fulfill Koch’s postulates.

Table 2. Origins of *Stagonosporopsis* isolates and GenBank accession numbers of ITS regions and *TUB* genes

Origin (city/county)	Isolate	Host	Accession no.		Species	Location ^a
			ITS	<i>TUB</i>		
Gochang	GC192-11	Watermelon	ON055719	ON093927	<i>Stagonosporopsis caricae</i>	h
Chuncheon	CC203-17	Muskmelon	ON055703	ON093933	<i>Stagonosporopsis citrulli</i>	j
Gwangju	GJ201-3	Muskmelon	ON055716	ON093945	<i>Stagonosporopsis</i> sp.	c
Goryeong	GR212-27	Muskmelon	ON055704	ON093934	<i>Stagonosporopsis citrulli</i>	m
Goryeong	GR212-29	Muskmelon	ON055705	ON093935	<i>Stagonosporopsis citrulli</i>	m
Goryeong	GR213-2	Muskmelon	ON055706	ON093936	<i>Stagonosporopsis citrulli</i>	m
Goryeong	GR213-3	Muskmelon	ON055707	ON093937	<i>Stagonosporopsis citrulli</i>	m
Goryeong	GR214-1	Muskmelon	ON055708	ON093938	<i>Stagonosporopsis citrulli</i>	m
Changwon	CW211-1	Muskmelon	ON055720	ON093928	<i>Stagonosporopsis caricae</i>	n
Changwon	CW211-2	Muskmelon	ON055709	ON093939	<i>Stagonosporopsis citrulli</i>	n
Changwon	CW211-3	Muskmelon	ON055710	ON093940	<i>Stagonosporopsis citrulli</i>	n
Changwon	CW211-11	Muskmelon	ON055711	ON093941	<i>Stagonosporopsis citrulli</i>	n
Changwon	CW214-3	Watermelon	ON055717	ON093946	<i>Stagonosporopsis</i> sp.	n
Chunan	CA215-1	Muskmelon	ON055721	ON093929	<i>Stagonosporopsis caricae</i>	e
Chunan	CA217-1	Muskmelon	ON055722	ON093930	<i>Stagonosporopsis caricae</i>	e
Yanggu	YG211-4	Watermelon	ON055718	ON093947	<i>Stagonosporopsis</i> sp.	i
Yanggu	YG211-5	Watermelon	ON055712	ON093942	<i>Stagonosporopsis citrulli</i>	i
Yanggu	YG211-10	Watermelon	ON055713	ON093943	<i>Stagonosporopsis citrulli</i>	i
Yanggu	YG211-13	Watermelon	ON055723	ON093931	<i>Stagonosporopsis caricae</i>	i
Yangpyeong	YP216-4	Watermelon	ON055714	ON093944	<i>Stagonosporopsis citrulli</i>	k
Yangpyeong	YP216-8	Watermelon	ON055724	ON093932	<i>Stagonosporopsis caricae</i>	k

ITS, internal transcribe spacer; *TUB*, β -tubulin; GSB, gummy stem blight.

^aGSB causal agents were not isolated from a, b, d, f, g, and l.

Results

Sampled locations and putative *Stagonosporopsis* isolates. Leaves and stems of watermelon and muskmelon displaying GSB symptoms were collected from 43 farms distributed in 14 cities of Korea (Table 2, Fig. 1A). Multiple fungal colonies were obtained from each symptomatic tissue (Fig. 1B) and only those with *Stagonosporopsis* morphologies were selected as isolates and cultured to homogeneity.

Identification of genus *Stagonosporopsis* based on ITS region. ITS region of nuclear ribosomal RNA has been used to identify *Stagonosporopsis* spp. (Choi et al., 2010, 2015; Stewart et al., 2015; Zhang et al., 2019). The ITS regions of putative *Stagonosporopsis* isolates were amplified with primers, ITS1 and ITS4 (Table 1, Supplementary Fig. 1), and the sequences of each amplicon were compared with the NCBI database using the blastn algorithm. Blast results of the ITS sequences demonstrated that a total of 21 isolates belong to the *Stagonosporopsis* genus (Table 2). Phylogenetic analysis based on the ITS region was con-

ducted with references retrieved from GenBank (5 from *S. cucurbitacearum*, 9 from *S. citrulli*, 3 from *S. caricae*, and 1 outgroup *S. heliopsisidis*) by maximum likelihood based on the Jukes-Cantor model using MEGA X software (Supplementary Fig. 2). Four Korean Agriculture Culture Collection (KACC) strains previously identified as *D. bryoniae*, KACC 40937, KACC 40669, KACC 40900, KACC 40939 were included in the phylogenetic analysis. Although *S. caricae* references were grouped into one clade, all other references including *S. cucurbitacearum* and *S. citrulli*, were not successful in dividing into different clades. These results suggest that sequences from the ITS region alone cannot clearly discriminate between the *Stagonosporopsis* species.

Classification of *Stagonosporopsis* species based on β -tubulin gene. The *TUB* gene has been also commonly used for inferring phylogenetic relationships among *Stagonosporopsis* spp. (Aveskamp et al., 2010; Stewart et al., 2015; Zhang et al., 2019). Therefore, after PCR amplification of *TUB* using a primer set, TUB-T1_F and TUB_T22_R (Table 1, Supplementary Fig. 3), *TUB* sequences from all isolates and four KACC strains and the references retrieved

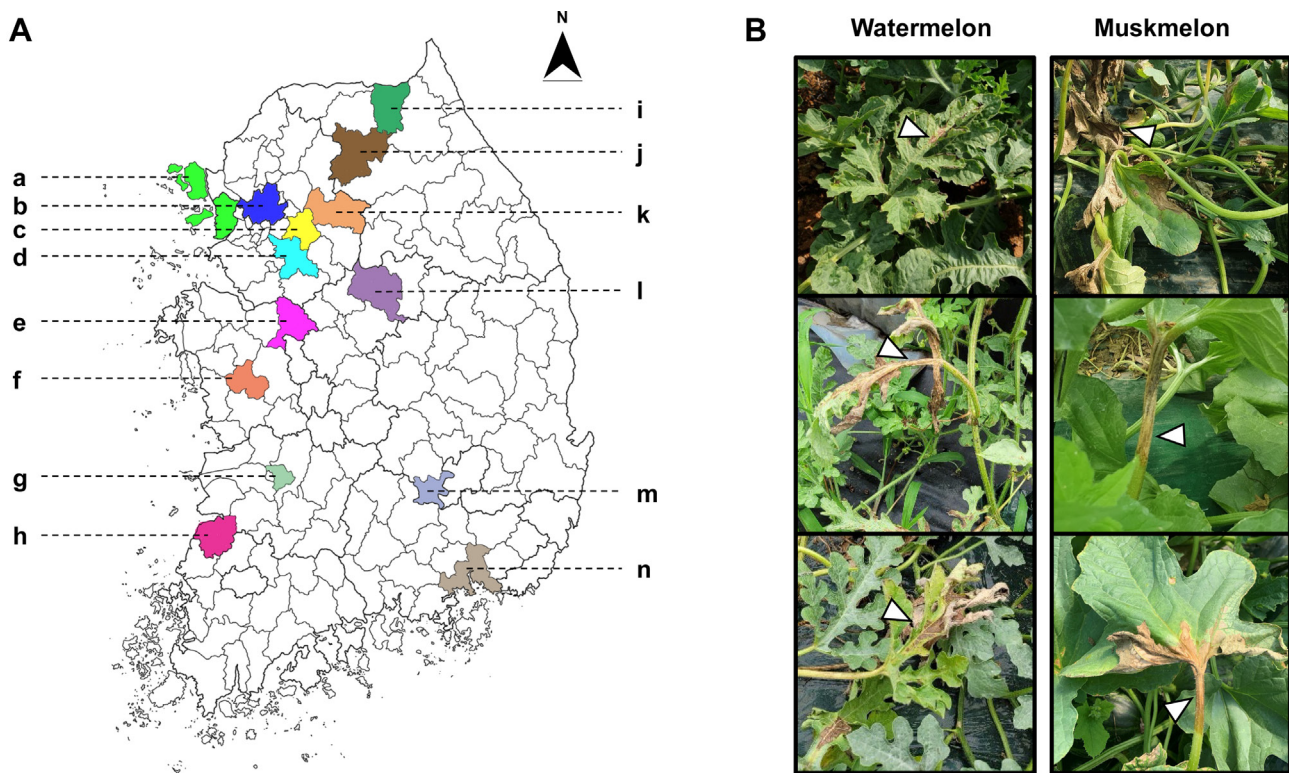


Fig. 1. Watermelon and muskmelon farm locations and representative gummy stem blight (GSB) symptoms on leaves and stems. (A) Map showing 14 cities and counties in Korea. (B) Typical GSB displaying brown discoloration and necrotic lesions on watermelon and muskmelon leaves and stems. Arrowheads indicate typical symptoms of GSB. GSB causal agents were not isolated from a, b, d, f, g, and l.

from GenBank were subjected to *TUB*-based phylogenetic analyses (Supplementary Fig. 3). All references in phylogenetic tree were placed within three different monophyletic clades with isolates together, indicating that the *TUB* gene is more useful than ITS region for assaying genetic diversity in the *Stagonosporopsis* spp.

Multi-locus analysis is often more useful for species classification and is successfully applied for many fungal pathogens (Aveskamp et al., 2010). Therefore, multi-locus phylogenetic analysis of ITS and *TUB* sequences from all *Stagonosporopsis* isolates and four KACC strains was conducted together with reference sequences retrieved from GenBank (Fig. 2). Twelve isolates, including YG211-10, YG211-5, GR213-3, GR214-1, GR213-2, GR212-29, GR212-27, CC203-17, CW211-2, CW211-3, CW211-11, and YP216-4, and four KACC strains previously identified as *D. bryoniae*, were located in one clade with reference *S. citrulli*. Six isolates, GC192-11, CW211-1, YG211-13, YP216-8, CA215-1, and CA217-1, were together with *S. caricae*. However, none of our isolates was located with *S. cucurbitacearum*. Interestingly, there was one clade consisting of three isolates, GJ201-3, YG211-4, and CW214-3, which did not belong to the three *Stagonosporopsis* spp.

known to cause GSB, suggesting that they could be an unknown species of *Stagonosporopsis* causing GSB in Korea.

Additional multi-locus phylogenetic analysis of ITS and *TUB* sequences was conducted using all 21 species of the genus *Stagonosporopsis* available in GenBank (Aveskamp et al., 2010; Brewer et al., 2015). The unknown species was accurately placed within the *Stagonosporopsis*. However, it belonged to none of species in the *Stagonosporopsis* genus, suggesting that the unknown species is a new species of *Stagonosporopsis* (Supplementary Fig. 4).

Molecular genetic differentiation of *Stagonosporopsis* spp. using microsatellite markers.

To further validate the species identification performed by multi-locus phylogenetic analysis, three PCR-based microsatellite markers, Db01, Db05, and Db06 (Brewer et al., 2015), were applied to all *Stagonosporopsis* isolates and four KACC strains (Fig. 3). In a previous report, *S. citrulli* displays three PCR amplicons of approximately 220, 280, and 360 bp produced by Db05, Db06, and Db01, respectively (Brewer et al., 2015). *S. cucurbitacearum* produces two PCR amplicons of approximately 220 and 270 bp Db05 and Db06, respectively. In *S. caricae*, one PCR amplicon is amplified approxi-

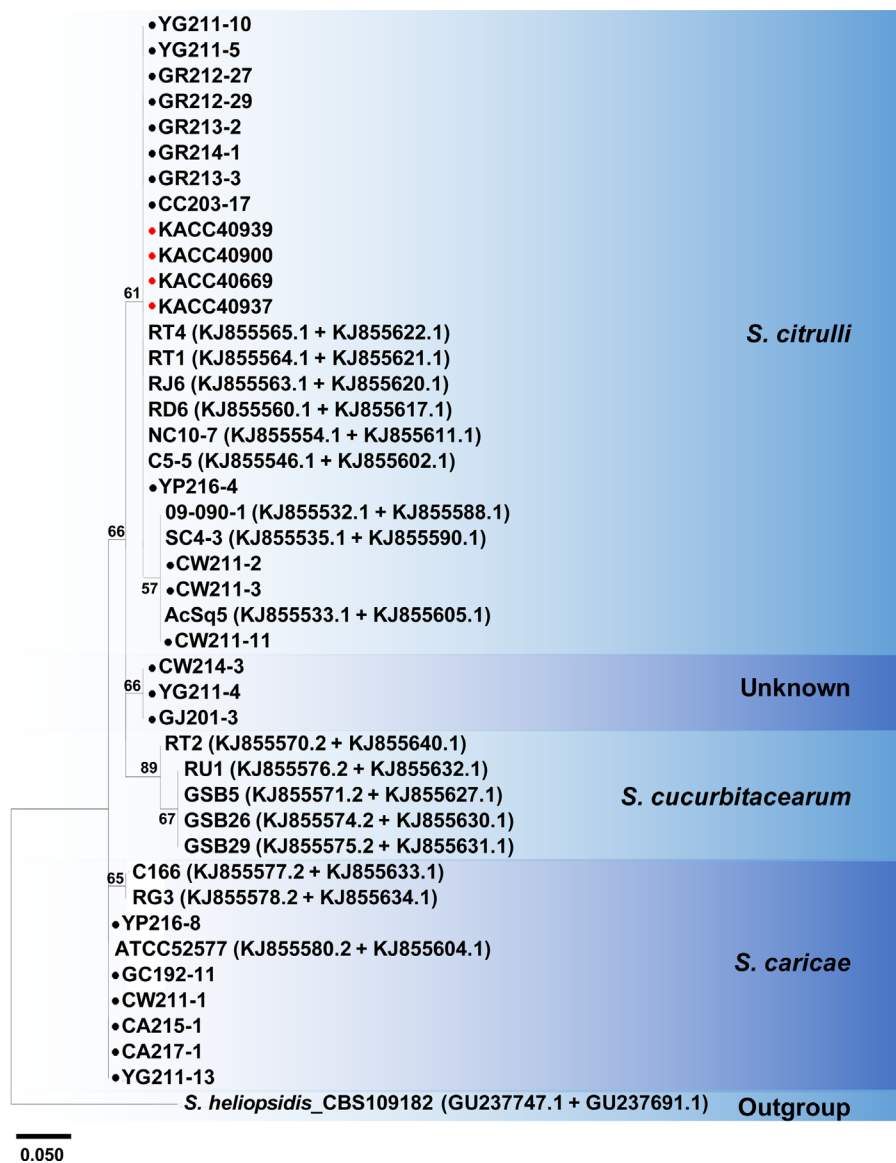


Fig. 2. Multi-locus phylogenetic analysis based on the internal transcribed space (ITS) and β -tubulin (*TUB*) genes. Twenty-one *Stagonosporopsis* isolates, four *Dydimella bryoniae* Korean Agriculture Culture Collection (KACC) strains, and 17 *Stagonosporopsis* reference (nine *S. citrulli*, five *S. cucurbitacearum*, and three *S. caricae*) were analyzed. All *Stagonosporopsis* reference sequences were retrieved from GenBank, and their GenBank accession numbers are shown after the name of each reference (ITS + *TUB*). Black circles indicate *Stagonosporopsis* isolates isolated in this study. Red circles indicate four *D. bryoniae* strains from KACC. Others without circles are references for *S. citrulli*, *S. cucurbitacearum*, and *S. caricae*. *S. heliopsisidis* was used as outgroup. For each branch, bootstrap support values obtained by maximum likelihood are indicated based on 500 replicates. Bootstrap support values less than 50% are not shown.

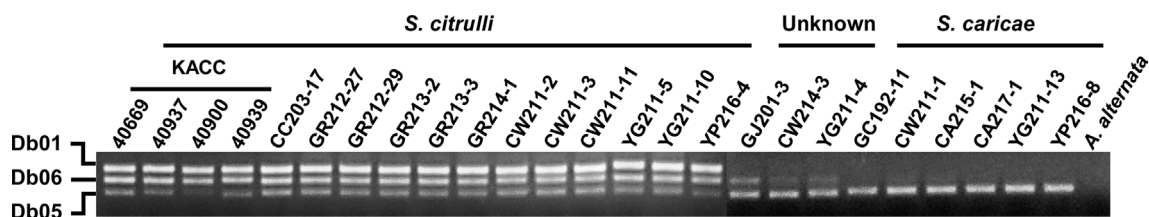


Fig. 3. Analysis of *Stagonosporopsis* isolates using microsatellite markers. Gel electrophoresis analysis after polymerase chain reaction using microsatellite markers, Db01, Db05, and Db06 (Table 1). Twenty-one *Stagonosporopsis* isolates and four *Dydimella bryoniae* Korean Agriculture Culture Collection (KACC) strains were analyzed. *Alternaria alternata* was used as a negative control.

mately 220 bp by Db05. In this study, all KACC strains and 12 isolates, including YG211-10, YG211-5, GR213-3, GR214-1, GR213-2, GR212-29, GR212-27, CC203-17, CW211-2, CW211-3, CW211-11, and YP216-4 produced three PCR amplicons, which is typical of *S. citrulli*. In six isolates, GC192-11, CW211-1, YG211-13, YP216-8, CA215-1, and CA217-1, single amplicon of without addi-

tional bands was amplified, confirming them as *S. caricae*. Three isolates, GJ201-3, YG211-4, and CW214-3, not grouped with three *Stagonosporopsis* spp., known to GSB causal agents, produced two amplicons.

To gain insight into variation within and among the three *Stagonosporopsis* spp. and further investigate the unknown species, we sequenced Db05 amplicons, which are only

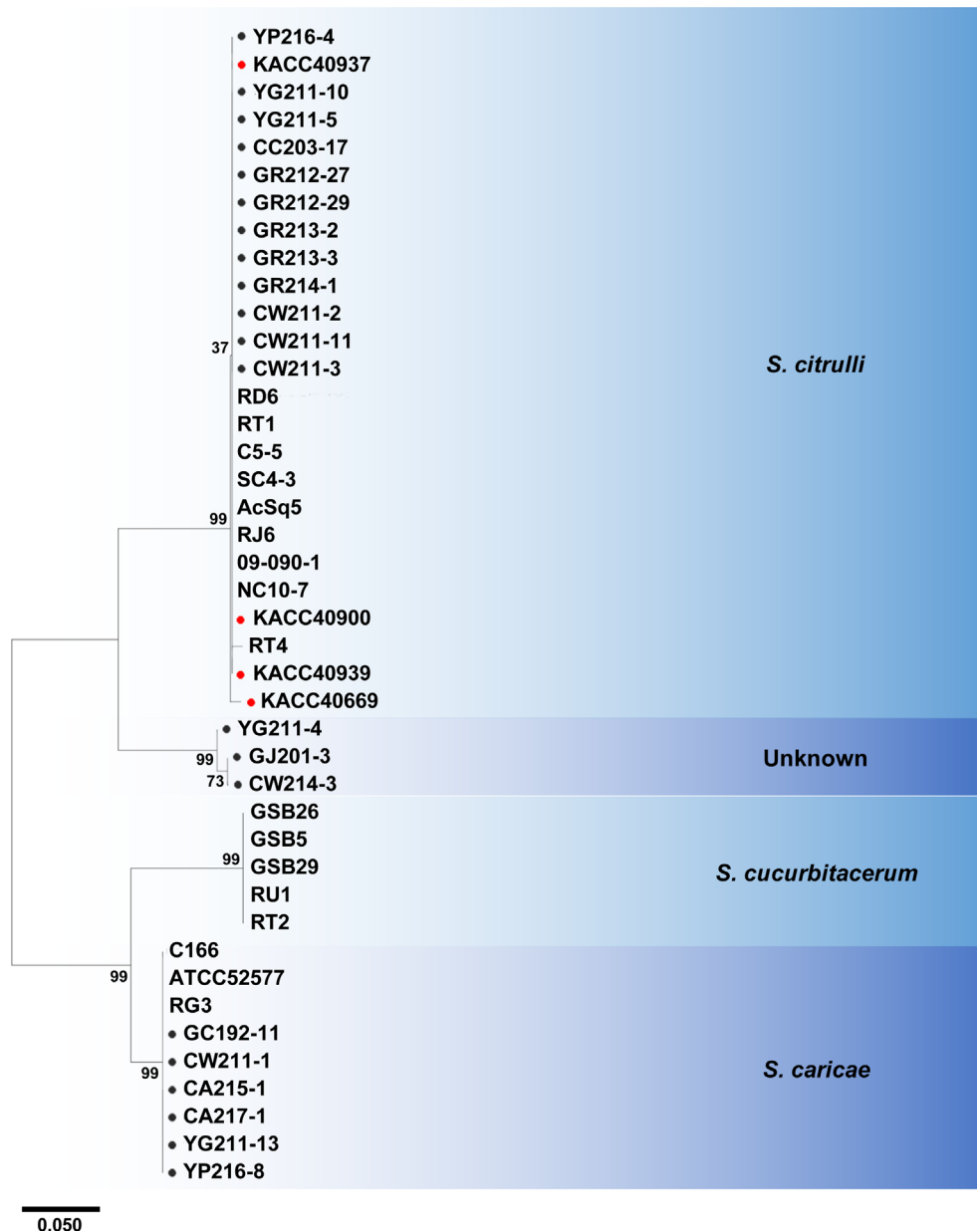


Fig. 4. Phylogenetic tree analysis based on Db05 sequence of 21 *Stagonosporopsis* isolates, four Korean Agriculture Culture Collection (KACC) strains, and 17 *Stagonosporopsis* reference (nine *S. citrulli*, five *S. cucurbitacearum*, and three *S. caricae*). Sequences of Db05 amplicons from *Stagonosporopsis* references were obtained from a previous study (Brewer et al., 2015). Black circles indicate *Stagonosporopsis* isolates isolated in this study. Red circles indicate KACC strains. Others are references for *S. citrulli*, *S. cucurbitacearum*, and *S. caricae*. For each branch, bootstrap support values obtained by maximum likelihood are indicated based on 500 replicates. Bootstrap support values less than 50% are not shown.

PCR products obtained from all isolates and KACC strains, and then compared them with references (Fig. 4). DB05 sequences of the references were retrieved from GenBank (Brewer et al., 2015). Similar to multi-locus phylogenetic analysis (Fig. 2), twelve and six isolates were together with *S. citrulli* and *S. caricae*, respectively. Again, three unknown species isolates, GJ201-3, YG211-4, and CW214-3, were clustered into one clade, while *S. cucurbitacearum* references form distinct clades of different evolutionary origin. In the Db05 alignment, there was no variation within *S. caricae* isolates and *S. caricae* reference RG3, and within *S. citrulli* isolates and *S. citrulli* reference 09-090-1 (Supplementary Fig. 5). There was only one insertion/deletion (indel) (4 bp) and one single nucleotide polymorphism (SNP) within unknown species isolates. However, the unknown species isolate (CW214-3) differed from *S. cucurbitacearum* (reference RT3) by 33 nucleotide polymorphisms (SNPs) and three indels (5, 7, and 6 bp). This

large diversity suggests that the unknown species is evolutionarily different from *S. cucurbitacearum*.

Morphological characterization and pathogenicity tests of *Stagonosporopsis* species.

Morphological characteristics of the 21 *Stagonosporopsis* isolates, including 12 *S. citrulli*, 6 *S. caricae*, and 3 unknown species isolates, were examined on PDA plates. Light-white aerial mycelium was observed on top and white to olivaceous on the bottom in all isolates (Fig. 5A). There was no obvious difference among each isolate except that isolates of *S. citrulli* presented slightly less intense mycelial growth compared to *S. caricae* and unknown species. Conidia obtained from each isolate were observed under a light microscope, and three representatives—CC203-17 (*S. citrulli*), GC192-11 (*S. caricae*), and GJ201-3 (unknown)—were further analyzed (Fig. 5B). The conidia were round-ended, cylindrical, monoseptate, and hyaline, and their length and diameter

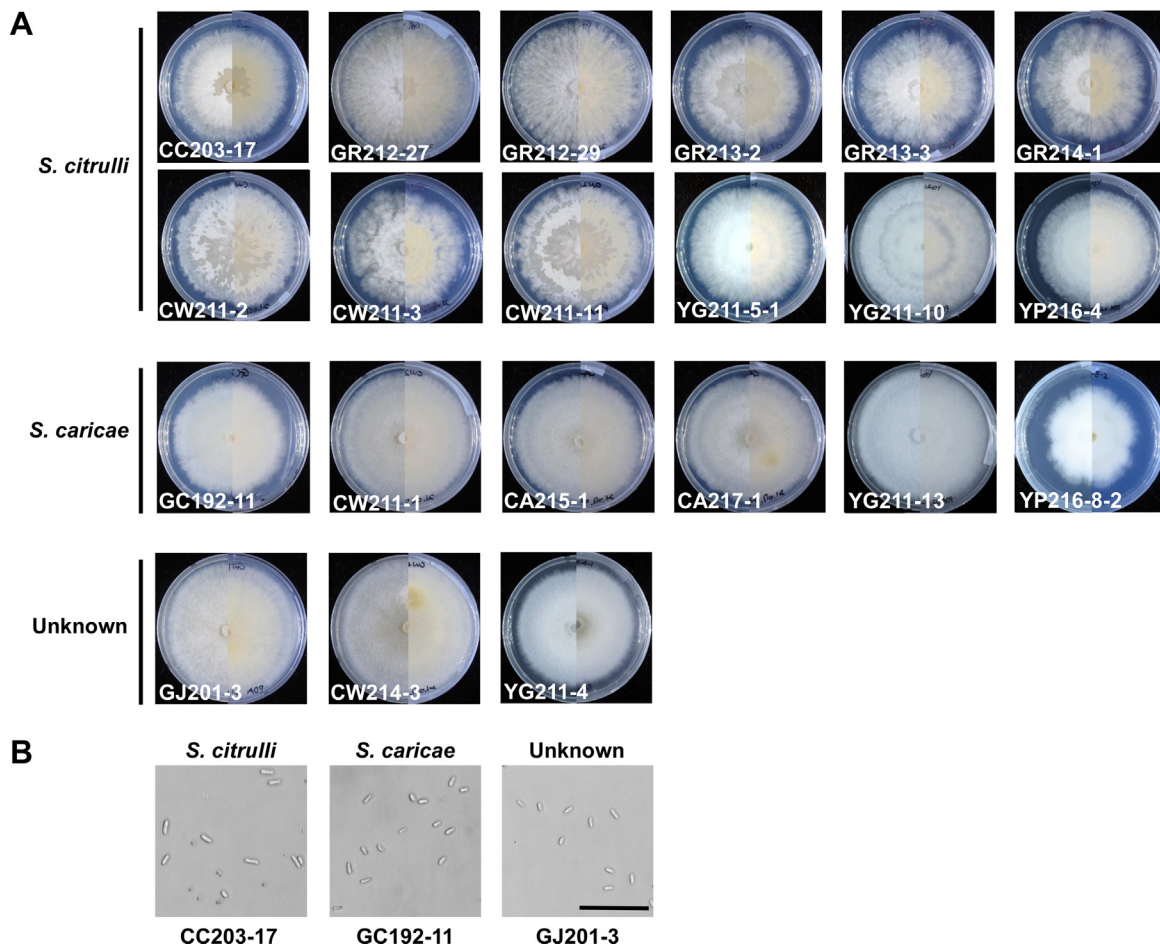


Fig. 5. Colonies of *Stagonosporopsis* isolates. (A) Pure cultures and morphological characteristics of all *Stagonosporopsis* isolates on potato dextrose agar (PDA) (front and reverse) 7 days after incubation at 28°C under continuous light condition. (B) Conidial morphology of *S. citrulli* GR213-3, *S. caricae* CW211-1, and unknown GJ214-3 on PDA media. Scale bar = 50 µm.

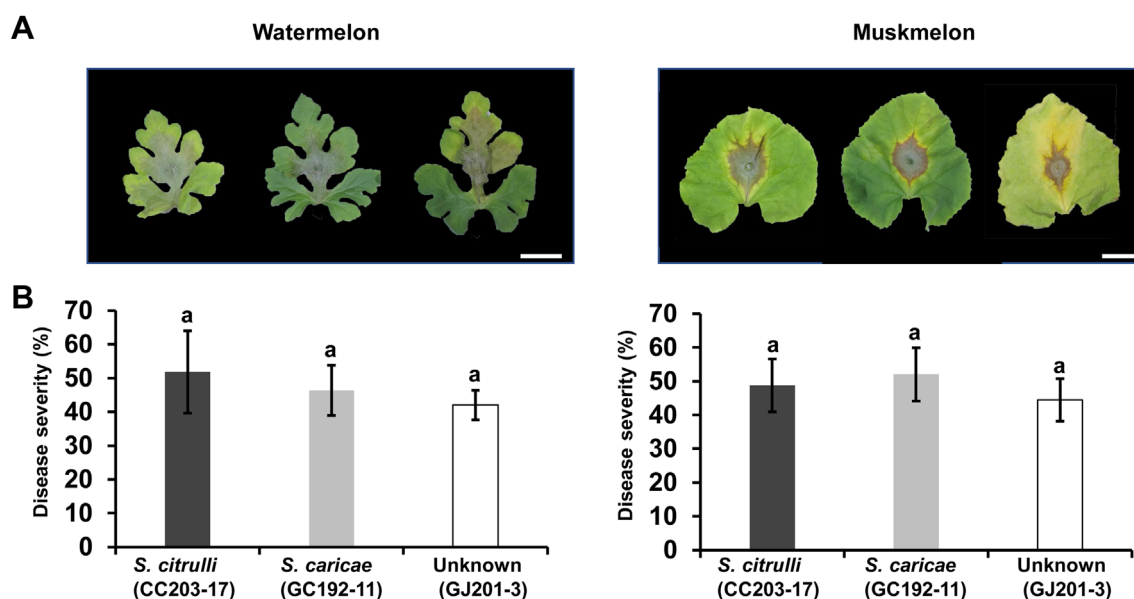


Fig. 6. Disease severity of three representative isolates of three *Stagonosporopsis* species on detached watermelon or muskmelon leaves. (A) One agar disk growing each *Stagonosporopsis* species was placed on the detached of watermelon cultivar ‘Seotaja’ and muskmelon cultivar ‘Earls top one’. Three leaves from each cultivar were inoculated, and images were obtained 7 days after inoculation. Scale bars = 3.0 cm. (B) Disease severity was calculated after each disease area was divided by whole leaf area using ImageJ. The data are the mean \pm SE of at least three independent replicates. The values with the same letter are not significantly different at $P < 0.05$.

ranged from approximately 3.5 to 11.1 and 1.2 to 3.9 μm , respectively.

The pathogenicity of isolates was evaluated on watermelon cultivar ‘Seotaja’ and muskmelon cultivar ‘Earls top one’ and disease symptoms caused by 14 representative isolates were presented (Supplementary Fig. 6). Visible symptoms of the leaves started 3 days after inoculation as dark brown lesions. The symptoms around the inoculation sites increased as blackish-brown water-soaked lesions. One week after inoculation, 100% of inoculated detached leaves of watermelon and muskmelon exhibited rapidly growing necrotic lesions with gray mycelium growth. The symptomatic tissues were cultured again on PDA, and the same pathogens were re-isolated confirming Koch’s postulates. Mock-inoculated leaves remained symptomless. To determine if the three species differed in aggressiveness, disease severity caused by three representative isolates—CC203-17 (*S. citrulli*), GC192-11 (*S. caricae*), and GJ201-3 (unknown)—from different species was quantified using ImageJ (Fig. 6). At 7 days after inoculation, there were no significant differences in aggressiveness of the three isolates on both watermelon and muskmelon.

Discussion

The genus *Stagonosporopsis* causing GSB in cucurbits has

been characterized relatively recently (Brewer et al., 2015; Stewart et al., 2015). *S. citrulli* was the most abundant species with the widest geographic distribution, and *S. caricae* was also distributed worldwide, whereas *S. cucurbitacearum* has been only found in temperate regions (Stewart et al., 2015). Given that causal agents of GSB have been reported as *D. bryoniae* until recently (Basim et al., 2016; Choi et al., 2010; Lee et al., 2016) and often misidentified as *S. cucurbitacearum* (Brewer et al., 2015), a careful study about the diversity and population structure of different GSB pathogens is necessary.

In this study, a multi-locus phylogenetic analysis of ITS and *TUB* sequences revealed that *S. citrulli* is the dominant species for the GSB and *S. caricae* is also relatively common in Korea. However, *S. cucurbitacearum* was not identified; instead, there was one clade consisting of three isolates, GJ201-3, YG211-4, and CW214-3, that did not belong to *Stagonosporopsis* spp., known to cause GSB. Additional multi-locus analysis conducted using all 19 species of the genus *Stagonosporopsis* available in GenBank displayed that although the three isolates accurately placed within the genus *Stagonosporopsis*, they were distinct from any previously known species of *Stagonosporopsis*. Our results were further confirmed using PCR-based microsatellite markers, Db01, DB05, and DB06. In addition, after sequence analysis of Db05 amplicons obtained from

all isolates and references, we found that there is great diversity between the three isolates and other previously known GSB causal *Stagonosporopsis* spp. Therefore, we concluded that the isolates are the previously unknown *Stagonosporopsis* species causing GSB on both watermelon and muskmelon.

It was previously reported that although differences in pathogenicity among three species—*S. citrulli*, *S. cucurbitacearum*, and *S. caricae*—to papaya was detected, there were no differences in the aggressiveness of the species on cucurbit hosts including watermelon, *Cucurbita moschata* (pumpkin), and cucumber (Stewart et al., 2015). We were also unable to determine the differences in aggressiveness among *S. citrulli*, *S. caricae*, and unknown *Stagonosporopsis* species, to watermelon cultivar ‘Seotaja’ and muskmelon cultivar ‘Earls top one’. However, additional further experiments with various potential hosts need to be performed, because they could vary in pathogenicity to other species and different cultivars.

In the study, we described *Stagonosporopsis* spp. infecting watermelon and muskmelon in Korea from 2019 to 2022. Pathogenicity tests with isolates of *S. citrulli* and *S. caricae* as well as unknown *Stagonosporopsis* species confirmed that all three species were able to cause GSB on watermelon and muskmelon, thereby completing Koch’s postulates. Correct identification of these pathogens will facilitate the management strategies to prevent damage caused by GSB in cucurbits.

Conflicts of Interest

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

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (<http://www.ppjonline.org/>).

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