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Isolation and identification of *Burkholderia gladioli* on *Cymbidium* orchids in Korea

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ABSTRACT

Bacterial diseases of *Cymbidium* orchids continue to threaten the floriculture industry. Bacterial isolates were isolated and cultured from *Cymbidium* plants exhibiting disease symptoms: black lesions with or without water-soaking on leaves. To identify the bacterial isolates, we performed polymerase chain reaction (PCR) amplification using species-specific primers. The pathogen was tentatively identified as *Burkholderia gladioli*, which was first described as a phytopathogen, and later associated with human infections, especially with patients with cystic fibrosis. PCR-restriction fragment length polymorphism analysis of 16S ribosomal DNA (rDNA) products digested with *Hinf*I and sequencing of the 16S rDNA confirmed the isolate as *B. gladioli*. *Burkholderia gladioli* strains infecting plants are divided into two pathovars: *alliiicola* and *gladioli*. Pathogenicity tests showed that the isolate proved to be pathogenic to *Cymbidium* orchid but not to onion plants, a common host for *B. gladioli* pv. *alliiicola*. To the best of our knowledge, this is the first report of *B. gladioli* in *Cymbidium* orchids in Korea.

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Cymbidium; onion; restriction
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Introduction

Burkholderia spp. is a genus of aerobic, Gram-negative, rod-shaped and motile proteobacteria found in diverse habitats. Some of them are reported to have plant growth-promoting, endophytic and antifungal strains with potential biocontrol activity [1]. However, many of them are mainly known as plant or human pathogens. One of them, *Burkholderia gladioli*, was initially identified as a pathogen of gladiolus [2]. It was subsequently reported that the bacteria are associated with diseases in other plants and three pathovars were identified according to their specialization to the host plant. *B. gladioli* pv. *alliiicola* (formerly known as *Pseudomonas alliiicola*) causes onion bulb rot, *B. gladioli* pv. *gladioli* (formerly *Pseudomonas marginata*) causes a leaf and corn disease in gladioli and irises and *B. gladioli* pv. *agaricicola* causes soft rot in the mushroom *Agaricus bitorquis* [3]. A fourth pathovar, *B. gladioli* pv. *cocovenenans* (formerly *Pseudomonas cocovenenans*), is a lethal toxin-producing strain and provides differential characteristics of the food-poisoning pathovar from phytopathogenic pathovars [4].



Orchidaceae (the orchid family) is a diverse and widespread family of flowering plants that often have fragrance and ornamental qualities. Orchidaceae is affected by a range of diseases, including root diseases, stem and pseudobulb decays, leaf spots and flower blights [5]. The

most common and important bacterial pathogens found in orchid farms include *Pectobacterium carotovora*, *Dickeya* spp., *P. cypripedii*, *Acidovorax cattleya* and *B. gladioli* [5,6]. Particularly, *B. gladioli* can cause serious losses in orchid production including Dendrobium, Oncidium, Miltonia and Cymbidium. For example, *B. gladioli* is one of the primary pathogens of orchids in Thailand, and the losses of Dendrobium orchids due to the bacterial disease have been estimated at 50% or more [6,7]. It has also been reported as a major pathogen of Dendrobium orchids in Hawaii as well [5].

Members of the genus *Cymbidium* belong to the orchid family and are popularly grown as potted flower plants in Korea. Many cultivars of *Cymbidium* with highly decorative flower spikes developed by breeding systems have been released on the domestic and international market. At present, *Cymbidium* occupies the largest cultivation area of any orchid in Korea, accounting for 47% (ca. 101 ha) of the total cultivated land for orchid production [8]. So far, the pathogens reported in Korea that cause *Cymbidium* disease include *Fusarium subglutinans*, *Sclerotium rolfsii*, *Acidovorax avenae*, *Cymbidium* mosaic virus and *Odontoglossum* ring spot virus [8–14].

Here, we describe the isolation and identification of the bacterial pathogen *B. gladioli* from symptomatic *Cymbidium* leaves based on microbiological

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characteristics, pathogenicity and the molecular approach. To the best of our knowledge, the finding of *B. gladioli* on the *Cymbidium* orchid is the first record in Korea, and we characterized the phenotypic and pathological properties of the isolate.

Materials and methods

Field survey and sampling

Samples were harvested from *Cymbidium* showing disease symptoms at nine farms located in various regions of South Korea (Figure 1). Samples were collected during farm visits and were kept at 4 °C until analysis.

Isolation and identification of the pathogen

For the isolation of casual agents, marginal sides of symptomatic areas from leaves were cut to approxi-

mately 1 cm² in size and sterilized with 70% ethanol for 1 min. The leaf tissues were placed on tryptic soy agar (TSA) plates, and then were incubated at 28 °C for two days. Bacterial colonies were streaked separately onto TSA plates for single colony isolation and were used for this study. The isolated colonies were maintained at 4 °C for short-term use or at –78 °C in a deep fridge for long-term storage in a glycerol stock. The reference strains *B. gladioli* pv. *gladioli* (KCTC 2967) and *B. gladioli* pv. *allicola* (KCTC 12374) [15], used as controls for comparison in the experiments, were obtained from the Korean Collection for Type Cultures (Jeongeup, South Korea).

Molecular characterization of bacteria

Polymerase chain reaction (PCR) amplification was carried out with species-specific primers in a C1000™ thermal cycler (BioRad, Foster City, CA, USA): CMG16-1 (5'-

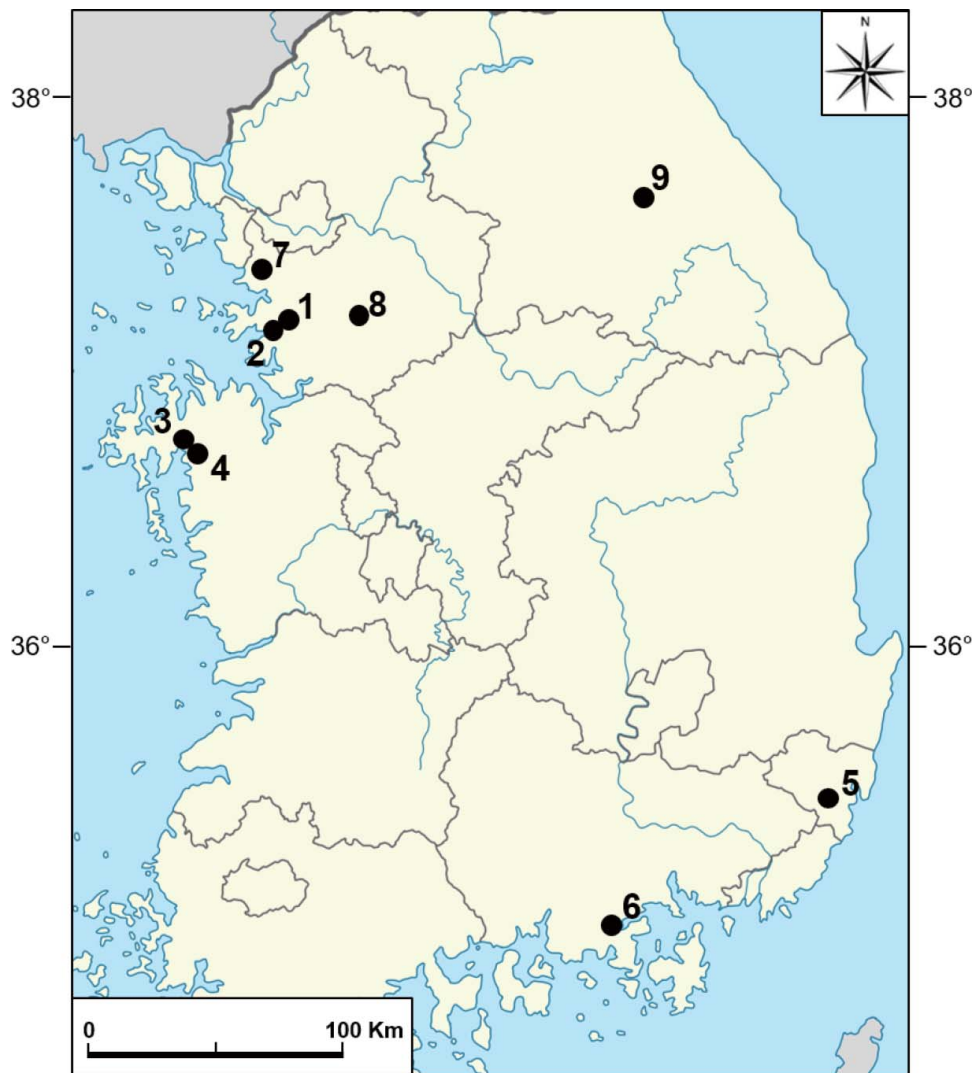


Figure 1. *Cymbidium* farm locations where samples were harvested in South Korea.

Note: 1: Hwaseong-si, Gyeonggi-do; 2: Hwaseong-si, Gyeonggi-do; 3: Seosan-si, Chungcheongnam-do; 4: Seosan-si, Chungcheongnam-do; 5: Ulju-gun, Ulsan; 6: Goseong-gun, Gyeongsangnam-do; 7: Siheung-si, Gyeonggi-do; 8: Yongin-si, Gyeonggi-do; 9: Pyeongchang-gun, Gangwon-do.

AGAGTTTGATCMTGGCTCAG-3') and G16-2 (5'-CGAAG-GATATTAGCCCTC-3') [16–18], GLA-f (5'-CGAGCTAA-TACCGCGAAA-3') and GLA-r (5'-AGACTCGAGTCAACTGA-3') [19]. PCR reaction was carried out in a 20 μ L final volume containing 1 \times PCR reaction buffer, 2.5 mmol/L of MgCl₂, 50 pmol of each primer, 0.1 mmol/L dNTP (deoxynucleoside triphosphate) mix, 0.5 U *Taq* DNA polymerase (Geneslabs, Seongnam, South Korea) and approximately 50 ng of bacterial genomic DNA extracted with ExiPrep™ Bacteria Genomic DNA Kit (Bioneer, Daejeon, South Korea). Amplification was performed with an initial denaturation step at 94 °C for 2 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min; and a final extension step at 72 °C for 7 min (T100™ Thermal Cycler, BioRad, Foster City, CA, USA). The amplified products were separated in 0.8% (w/v) agarose gel in 1 \times TBE (Tris-borate-ethylenediaminetetraacetic acid) buffer, stained with RedSafe™ dye (INTRON Biotech., Seongnam, Korea) and visualized under ultraviolet light. PCR products were cloned into the pGEM T-Easy Vector (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Plasmid DNA for sequencing was prepared with the PrimePrep plasmid DNA isolation Kit (GeNetBio, Nonsan, South Korea) according to the recommendations of the manufacturer. Nucleotide sequences from DNA sequencing were aligned using the ClustalW method of MegAlign software (Lageregene, Madison, WI, USA) and homology was determined using the National Center for Biotechnology Information (NCBI) Blast Network Server [20].

Restriction fragment length polymorphism (RFLP) analysis was performed with the restriction endonuclease *Hinf*I (New England Biolab) on nearly full-length 16S ribosomal DNA (rDNA) (approximately 1500 bp) amplified with primers fD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3') [21–23]. *B. gladioli* pv. *gladioli* (*Bgg*, KCTC 2967) and *B. gladioli* pv. *allii* (*Bga*, KCTC 12374) were used as reference controls. Nucleotide sequences of PCR products obtained from isolates 9 and 14 from *Cymbidium* farm No. 2, *Bgg* and *Bga* were aligned using the ClustalW method of MegAlign software (Lageregene) and homology was determined using the NCBI Blast Network Server (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) [20].

Koch's postulates

Bacteria grown on TSA plates for two days were used for the inoculation tests. To fulfil Koch's postulates, leaves from six-month-old *Cymbidium* were inoculated by wounding with a sterile toothpick smeared with a

reference control *Bga* and the isolate 2-9 obtained from *Cymbidium* orchids with disease symptoms. Sterile water was used for negative control inoculation. The inoculated *Cymbidium* plants were maintained in a growth chamber at 24 °C and 70% relative humidity for two weeks. Disease development was observed beginning at two to three days after inoculation. Bacteria were then reisolated from the *Cymbidium* leaves displaying symptoms and were grown on TSA plates at 24 °C for two days.

Pathogenicity tests

The ability of the isolate 2-9 to cause rot on a possible alternative host, onion, was determined. Overnight cultures of bacterial strains were scraped off TSA plates, suspended in sterile water at about 5.0×10^8 cfu/mL, and 10 μ L of the bacterial suspension was used as inoculum. Onion bulbs and slices of onion approximately 1.25 cm thick were placed in Petri dishes containing moistened 3M filter paper (Whatman, Maidstone, UK). Onion bulbs and scales were stabbed with a sterilized needle, and a 5 μ L droplet of the bacterial suspension was placed on the wounded site. Sterile water was used as a control inoculation. The inoculated bulbs and scales were kept in a growth chamber at 28 °C for 10 days. A necrotic zone or soft rot lesion at least 2 mm beyond the inoculation site was considered as evidence of infection for all pathogenicity tests. Bacteria were reisolated from marginal sides of the infected areas to confirm the identity of the pathogen. For onion leaf inoculation, at least five leaves of each onion plant were inoculated by the scissors-clipping method [24–26]. All inoculation experiments were conducted at least three times.

Results and discussion

Symptoms and isolation of the bacterium

Bacteria responsible for disease symptoms were recovered from *Cymbidium* farms located at various regions of South Korea (Figure 1). The isolates were identified by PCR amplification using species-specific primers. Previously, one of the species-specific primer set, CMG16-1 and G16-2, was developed to detect the presence of the specific gene regions in the area coding 16S rDNA of *B. gladioli* [16–18,27]. We used the primer set and successfully amplified an approximately 470-bp fragment of 16S rDNA from *B. gladioli* pv. *allii* (*Bga*) and *B. gladioli* pv. *gladioli* (*Bgg*) (Figure 2(A)). The amplicon was not observed from *Erwinia cypripedii*, *Dickeya chrisanthemi*, *Agrobacterium tumefaciens* or *Escherichia coli*, indicating that the primer set, CMG16-1 and G16-2, is species-specific. Two bacterial isolates (strain 2-9 and 2-14) from 18

of the diseased *Cymbidium* tissues from greenhouse No. 2 in the Kyonggi-do region gave amplification typical for *B. gladioli* (Figure 2(B)). Bacterial isolates from other farms did not produce specific amplification for *B. gladioli* (data not shown). *Bga* was used as a positive control (P). In PCR with another set of species-specific primers for *B. gladioli*, GLA-f and GLS-r [19], an approximately 300 bp fragment was amplified in strain 2-9 as well as in the positive controls *Bga* and *Bgg* (Figure 2(C)). These results indicate that *Cymbidium* displaying disease symptoms on leaves was infected by *B. gladioli*.

The recognized symptoms from infected leaves included dark-brown to black lesions often surrounded by water-soaking (Figure 3(A)). Bacterial isolates from this type of diseased *Cymbidium* leaf tissue revealed the presence of a dominant form of bacterial colonies on TSA. The isolated colonies produced round-shaped, beige-yellowish colonies, approximately 2–2.5 mm in diameter for two-day cultivation and with the majority of the colonies secreting a diffusible yellow or tan pigment into the media (Figure 3(B)).

Identification of *Burkholderia gladioli*

To examine the species-specific amplicons obtained by PCR using primer CMG16-1 and G16-2, we sequenced the 470-bp PCR products of isolate 2-9, 2-14, *Bga* and *Bgg*. The sequence from isolate 2-9 was identical (100%, without gaps) with the one from 2-14 (data not shown).

Their nucleotide sequences were compared with gene sequences of known strains in the GenBank sequence database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). A BLAST search supported the species-specific PCR results that the isolates were *B. gladioli* (data not shown) [28,29]. A sequence alignment of the gene products was constructed using MegAlign software (ClustalW method) (Figure 4). The alignment displayed only three mismatched nucleotides between *Bga* and *Bgg*. The nucleotide sequence from the isolate 2-9 displayed 100% and 99.4% identity with *Bga* and *Bgg*, respectively, demonstrating that the bacterial pathogen from diseased *Cymbidium* tissues is *B. gladioli*.

Based on the sequence analysis of PCR products (Figure 4), the bacterial isolates obtained from diseased *Cymbidium* samples could be ascribed to *B. gladioli*. Additionally, we carried out PCR-RFLP analysis of the isolates 2-9 and 2-14 with reference controls, *Bgg* and *Bga*. Nearly full-length 16S rDNA was amplified with primers fD1 and rD1. The RFLP patterns of bacterial isolates 2-9 and 2-14 obtained using *Hinf*I restriction endonuclease were the same as that of *Bgg* but not the *Bga* reference strain used in this study (Figure 5). Both strands of the PCR products were sequenced using primers fD1 and rD1 and then aligned using MegAlign software (Clustal W method) (Figure S1 in the Online Supplementary Appendix). Bacterial isolate 2-9 showed very high levels of similarity with both reference controls, *Bgg* and *Bga* (99.7%–99.8%).

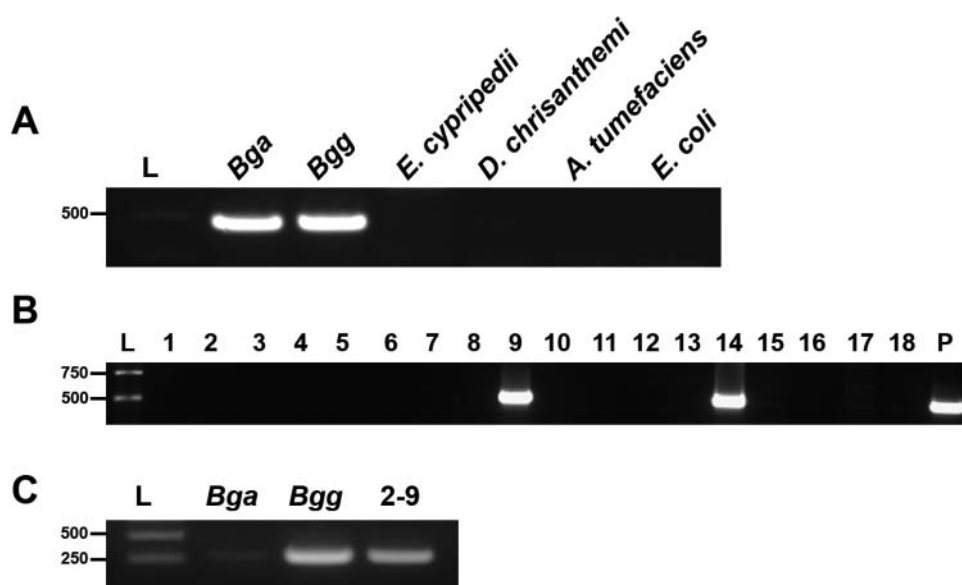


Figure 2. Detection of *B. gladioli* from *Cymbidium* samples harvested from *Cymbidium* farm No.2. (A) Amplification using species-specific oligonucleotide primers, CMG16-1 and G16-2, to detect *B. gladioli*. L: size marker (Geneslabs, Seongnam, South Korea). Note: Non-related bacteria, *E. cypripedii*, *D. chrysanthemi*, *A. tumefaciens*, *E. coli*, were used as controls to test nonspecific PCR amplification. (B) Detection of *B. gladioli* in leaf tissue samples (9 and 14). L: size marker; 1–18: *Cymbidium* samples harvested from *Cymbidium* farm No.2; P: positive control for *B. gladioli*. (C) Amplification using species-specific primers, GLA-f and GLS-r, for *B. gladioli*. Positive controls, *B. gladioli* pv. *galioli* (*Bga*) and *B. gladioli* pv. *galioli* (*Bgg*); L: size marker; 2-9: bacterial isolate 2-9.



Figure 3. Natural symptoms of bacterial leaf rot caused by isolate 2-9 on *Cymbidium* orchids (A) and colony morphology of *Burkholderia gladioli* pv. *galioli* (*Bga*), *Burkholderia gladioli* pv. *galioli* (*Bgg*) and isolate 2-9 (B).

Reisolation of *Burkholderia gladioli* from inoculated *Cymbidium* orchids

Six-month-old *Cymbidium* plants were artificially inoculated with isolate 2-9 and *B. gladioli*, and lesion development was monitored and compared for 12 days after inoculation (Figure 6). Negative control with a toothpick displayed a small wounding site without further development for 12 days. However, inoculation with isolate 2-9 and *B. gladioli* resulted in development of lesions around the inoculation sites with black necrosis. The water-soaked lesions appeared three to eight days after inoculation, rapidly enlarged and turned to dark-brown to black lesions within 8 to 12 days after inoculation. Eventually, the symptoms became similar to those observed in the greenhouse as shown in Figure 2(A). Reisolations from the artificially inoculated *Cymbidium* plants produced

round-shaped beige-yellowish colonies of approximately 2–2.5 mm in diameter for two-day cultivation similar to the colonies in Figure 2(B) (data not shown). Identification of the bacterium was confirmed by PCR using *B. gladioli*-specific primers, CMG16-1 and G16-2, followed by sequencing analysis, thus fulfilling Koch’s postulates (Figure S2 in the Online Supplementary Appendix).

Pathogenicity tests

Plant strains of *B. gladioli* are divided into pathovars, *allii-coli* and *gladioli*, according to their specialization to the host plant [30,31]. According to the early work by Ballard et al. [32], pv. *allii-coli* and pv. *gladioli* are indistinguishable using physiological testing. Studies on the distinction between the pathovars of *B. gladioli* are nearly non-



Figure 4. Sequence alignment of 470-bp fragment of the 16S rDNA of the isolate 2-9, *B. gladioli* pv. *galioli* (*Bga*), *B. gladioli* pv. *galioli* (*Bgg*).

Note: The gene products amplified with primers CMG16-1 and G16-2 were constructed using MegAlign software (Clustal W method).

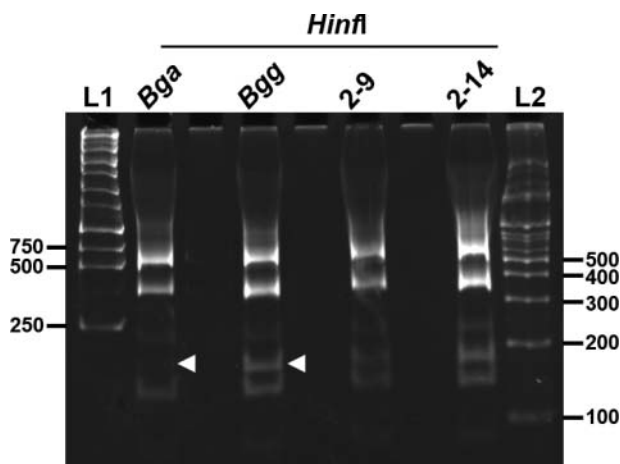


Figure 5. Identification of isolates 2-9 and 2-14 by PCR-RFLP analysis. L1: 1 kb size marker (Geneslabs, Seongnam, South Korea); L2: 100 bp size marker (Genepia, Seoul, South Korea). Note: Amplification with primers fD1 and rD1; digestion with *HinfI* restriction endonuclease. *B. gladioli* pv. *galioli* (*Bga*), *B. gladioli* pv. *galioli* (*Bgg*).

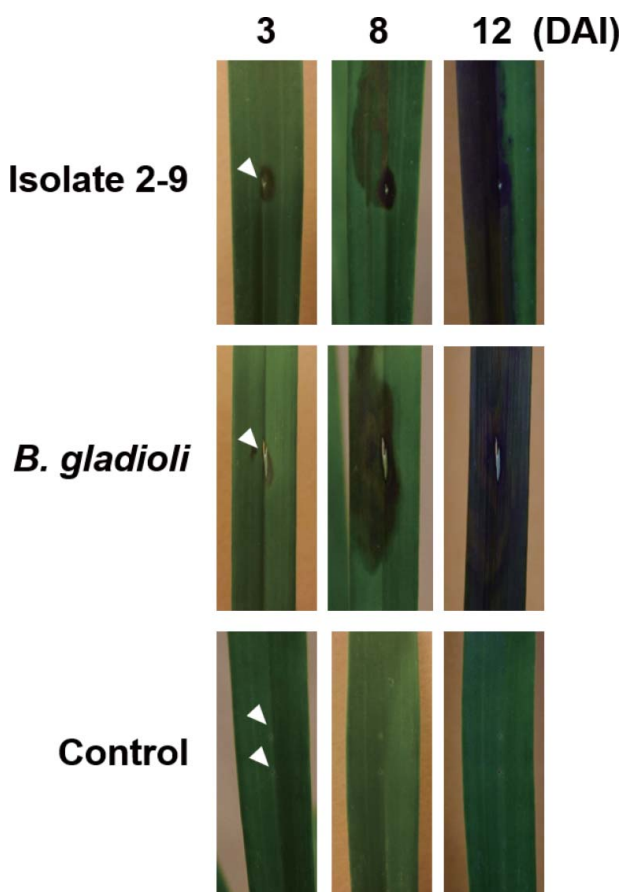


Figure 6. Symptom development of bacterial leaf rot in *Cymbidium* plants experimentally infected with isolate 2-9 and *B. gladioli* pv. *alliicola* (*B. gladioli*). Note: Images obtained at 3, 8 and 12 days after inoculation (DAI). Control inoculation was with sterile water. White triangles indicate punching sites on the *Cymbidium* leaves with a sterile toothpick for inoculation.

existent [17,18]. In the present study, the isolate 2-9 was identified as *B. gladioli* by PCR analysis using species-specific primers and the sequence analysis of the 16S rDNA gene. *Bga* and *Bgg* are very closely related and indistinguishable phylogenetically and phenotypically [30–32]. It was difficult to determine the pathovar of the isolate 2-9 by molecular analysis including PCR-RFLP and 16S rDNA sequencing because of high similarity between pathovar *alliicola* and pathovar *gladioli*. Therefore, we decided to perform a pathogenicity test using a possible alternative host.

Bgg has very wide host ranges including gladioli, freesia, irises and *Dendrobium* [1]. On the contrary, *Bga*, which infects tulip and onion [1], is well known to cause ‘slippery skin’ on onion bulbs and dry necrotic spots on onion leaves [15,17,18]. The first report in Korea of soft rot of onion by *Bga* was in the summer of 1999 [15], and it is now becoming a common disease during storage or transit. Previously, 25 isolates of *B. gladioli* were characterized and 10 of the isolates were identified as *B. gladioli* pv. *gladioli* in Italy, since none of them was pathogenic on onion plants [21]. To obtain more precise identification of the 2-9 isolate, we carried out a pathogenicity test using onions. For the pathogenicity assay, onion bulbs were inoculated with isolate 2-9, *Bgg* and *Bga*. After inoculation and incubation at 28 °C for 10 days, photo images were obtained from the cross section and the scale of inoculated onion bulbs. The tissue around the point inoculated with *Bga* became bright yellow and water-soaked 10 days after inoculation (Figure 7). On the contrary, onion bulbs inoculated with *Bgg* or isolate 2-9 displayed dried wound in the absence of typical symptoms of *B. gladioli*, water-soaked and macerated internal rot. Detached onion bulb scales inoculated with isolate 2-9, *Bgg* or *Bga* also displayed similar results (data not shown). When 20-fold concentrated bacterial inoculums were applied, only onion bulbs inoculated with *B. gladioli* pv. *alliicola* were macerated at 10 days after inoculation (Figure S3 in the Online Supplementary Appendix).

To quantify the pathogenicity of *B. gladioli* and isolate 2-9 on onion plants, the leaf tips of onion plants were inoculated with *Bga*, *Bgg* and isolate 2-9 using the scissors-clipping method [24,25]. The disease progress of *Bga* was rapid from the tip of the leaf, resulting in the wilting and eventual drying of the infected leaves (Figure 8(A)). At two days after inoculation, significant increases in the lesion lengths were detected in onions inoculated with *Bga* compared with those infected by *Bgg* and the isolate 2-9 (Figure 8(B)). At 14 days after inoculation, *Bga* displayed significantly larger lesion lengths of around 2.5 cm compared to *Bgg* and the isolate 2-9 displaying 0.5 cm lesion lengths similar with the negative control.

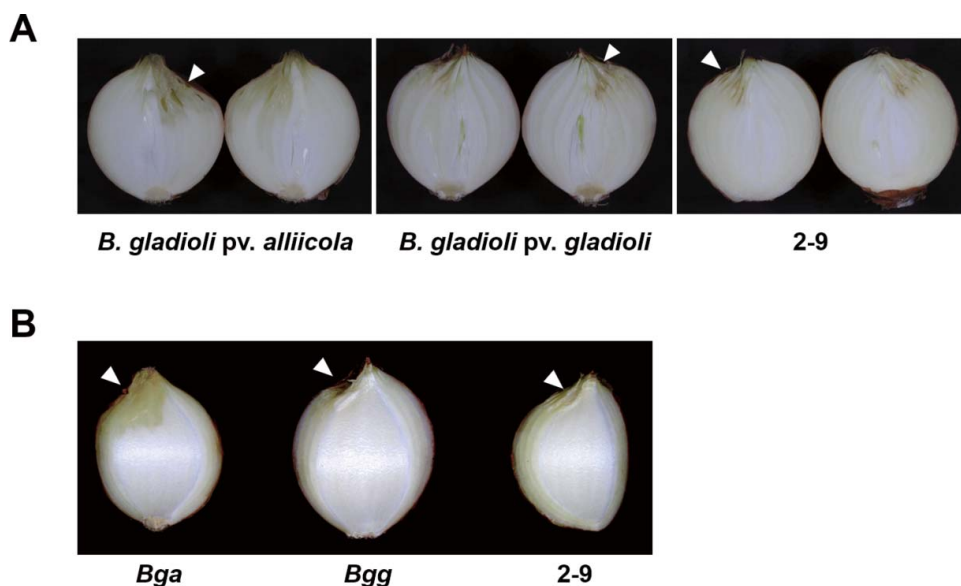


Figure 7. Symptoms of cross-inoculation studies of isolate 2-9 on a possible alternative host, onion, 10 days after inoculation. Onion bulbs (A) and scales (B).

Note: White triangles indicate regions inoculated with toothpicks. (A) Onion bulbs were inoculated with 10 μ L of *B. gladioli* pv. *alliicola* (*Bga*), *B. gladioli* pv. *gladioli* (*Bgg*) and isolate 2-9 at 5.0×10^8 cfu/mL. (B) Onion scales obtained from (A) were imaged for typical internal rot.

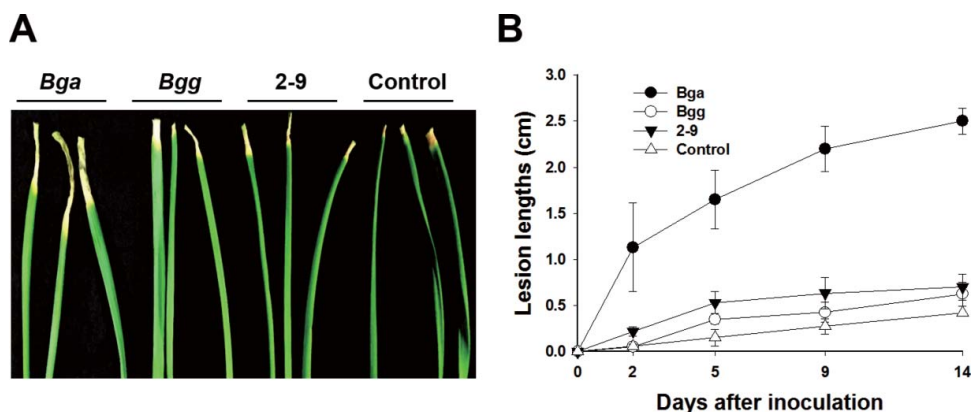


Figure 8. Symptom development on onion leaves inoculated with *B. gladioli* pv. *alliicoli* (*Bga*), *B. gladioli* pv. *gladioli* (*Bgg*) and isolate 2-9. Onion leaves (A) and lesion length measurements (B) of bacteria-inoculated plants over 14 days.

Note: Each data point represents the average and standard deviation of at least five samples.

Taken together, our results indicate that the isolate 2-9 is pathogenic to *Cymbidium* but not to onion, suggesting that the isolate 2-9 could be *Bgg*. However, although onion plants are common host plants for *Bga*, there are a few reports that *Bgg* is isolated from diseased onions [17–19]. Therefore, although isolate 2-9 is most likely *Bgg*, the interpretation of the results from the pathogenicity test should be viewed with caution. Because bacterial rot disease on *Cymbidium* caused by *B. gladioli* may have an extensive economic impact and quarantine on the *Cymbidium* industry, additional nation-wide monitoring should be carried out in Korea. In addition, copper and streptomycin resistance is widespread among strains of *B. gladioli* in nurseries [5]. It has been suspected that frequent use of chemicals containing copper and

streptomycin may have inadvertently resulted in the development of the resistant *B. gladioli* [5]. Therefore, in addition to a monitoring system of *B. gladioli*, adequate attention to control the bacterial disease is required to minimize economic losses in the *Cymbidium* industry.

Conclusions

In this study, bacterial isolates were isolated from *Cymbidium* leaves exhibiting dark-brown to black lesions. The isolated colonies produced round-shaped, beige-yellowish colonies for two-day cultivation and secreted a diffusible yellow or tan pigment into the media. Sequence analysis of nearly full-length 16S rDNA displayed very high levels of identity with two pathovars of

B. gladioli, *Bgg* and *Bga*. To achieve pathovars determination of the isolate, we performed a pathogenicity test using a possible alternative host, onion. Bulbs and leaves of onion plants inoculated with the isolate and a reference strain *Bgg* displayed dried wound in the absence of typical symptoms of *Bga*, water-soaked and macerated internal rot. Our results suggest that dark-brown to black lesions of *Cymbidium* plants are caused by *Bgg*. This is, to the best of our knowledge, the first description of occurrence of bacterial rot of *Cymbidium* plants caused by *B. gladioli* in Korea.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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