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Pathogenic variability of *Colletotrichum* species on different hot pepper varieties in South Korea based on nrDNA ITS region

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ABSTRACT

Hot pepper is usually attacked by various pathogens, which can cause large production loss. Molecular identification is the most commonly used method to discriminate the species of pathogens, plants, and even animals. Among the numerous DNA markers, the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) region was one of the most widely used markers for pathogen species systematics. In this study, we collected 26 pepper materials from different geographical regions in South Korea, and checked and identified the pathogen species they carried based on the sequence analysis result of the nrDNA ITS region. According to the sequence analysis analogue with the existing sequence reports in the NCBI GenBank database, there were five anthracnose *Colletotrichum* species found in 26 materials in all, including *C. acutatum*, *C. scovillei*, *C. gloeosporioides*, *C. nymphaeae*, and *C. simmondsii*. Among them, *C. scovillei*, considered the *C. acutatum* species complex, combined with *C. acutatum* and *C. gloeosporioides*, were considered as the most common anthracnose pathogens in peppers, while *C. nymphaeae* and *C. simmondsii* were firstly reported to infect peppers in South Korea, although these two species had been found to infect other vegetables, fruits, and other plant species. The effective advanced detection of potential pathogens would help to direct disease prevention and treatment.

Key words: *Capsicum annuum*, *Colletrotrichum nymphaeae*, *Colletrotrichum simmondsii*, anthracnose pathogen, pathogenic diversity.

INTRODUCTION

Hot pepper (*Capsicum annuum* L., family Solanaceae) is an important vegetable crop grown in almost all tropical and subtropical regions of the world (Guan *et al*., 11). Due to being rich in vitamin A and C, and having biting pungent flavour, pepper has been considered as a cash crop (Asgar *et al*., 2). However, during the whole growth period of this crop, and even post-harvest, handling, drying, transportation, and storage processes, pepper is usually threatened in various biotic stresses (Mishra *et al*., 15). The most serious impact for crop production and quality is infecting by some destructive pathogens. Some of them have been proved to be capable to cause human ill, while some have no direct pathogenicity evidence. For instance, pepper mild mottle virus, belonging to the genus *Tobamovirus*, has been associated with the clinical symptoms such as fever, abdominal pains, and pruritus (Colson *et al*., 6). These pathogens are usually extremely stable in the environment and could be transmitted by contact, such as tobacco mosaic virus. Until now, there is no effective approach to prevent pepper seedlings from these virus diseases or pathogen infection. Even some peoples seem that host resistance breeding is the only effective approach toward development of virus disease resistance in pepper (Mishra *et al*., 15). Thus, to seek an effective, economical, environment, friendly and durable control strategy to control these virus diseases or pathogens becomes urgent and necessary.

To provide an effective approach to control different pathogens and treat the infected pepper seedlings, the understanding of the species identification of pathogens is very important, and this work is the basis toward the development of prevention and cure methods of various pathogens. Molecular identification based on DNA markers is the commonly used method for the systematic of plants, fungi, and even animals. Due to high primer universality and efficient amplification, the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) is the most widely used target region in plant and fungi systematic (Álvarez and Wendel, 1).

In this study, we analyzed the number and species of potential pathogens on different healthy pepper varieties in different geographical regions in Korea. Five anthracnose *Colletotrichum* species, including *C. acutatum*, *C. scovillei*, *C. gloeosporioides*, *C.*

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nymphaeae, and *C. simmondsii* have been found from these pepper materials. Among these five anthracnose pathogen species, *C. acutatum* has been described as one of the most destructive pathogens of chilli worldwide (Cannon *et al*., 4; Wharton and Dieguez-Uribeondo, 19). This species has been found as anthracnose pathogen of peppers in South Korea (Han *et al*., 12), and combined with the other species, *C. gloeosporioides*, these both *Colletrichum* species have been commonly found in peppers in the Southeast Asian region (Than *et al*., 16). *C. gloeosporioides* has been found to infect peppers in the Philippines (Dela-Cueva *et al*., 7). However, the other both *Colletrichum* species, *C. nymphaeae* and *C. simmondsii* have not been found in the infection of peppers, although there were few reports about the infection of fruits, other vegetables and other plant species. This is the first report about the infection situation of *C. nymphaeae* and *C. simmondsii* in peppers in South Korea. As *Colletotrichum* species could induce severe production loss of pepper and other plant species, this work could provide methods and basis for early detection, prevention and treatment of pepper diseases, and direct the method development of targeted, effective pathogen control.

MATERIALS AND METHODS

Plant materials

Twenty-six pepper materials investigated in this study were collected from different geographical regions in South Korea (Table 1). The fresh mature leaves were sampled from these pepper materials and immediately stored in liquid nitrogen conditions until DNA isolation was performed. The collected pepper seedlings were all seemed to be healthy.

DNA isolation, PCR amplification and sequencing

DNA isolations were performed by using the modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 8). The ITS1-5.8S-ITS2 region was amplified using universal primers ITS1 and ITS (White *et al*., 33) in 20 µl PCR reaction. PCR was performed using a Gene Amp 9700 PCR system (Applied Biosystems Incorporate, Warrington, Cheshire, UK) in 20 µl volumes with the following reaction components: 1 µl of template DNA (~ 1-100 ng), 10 × Ex Taq Buffer (TaKaRa Bio Inc., Japan), 200 µmol l^{-1} each dNTP, 0.1 µmol l^{-1} of each primer, and 0.1 µl of TaKaRa Ex Taq (5 units μl^{-1}, TaKaRa Bio Inc., Japan). The PCR protocol included an initial denaturation step of 94°C for 1 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, extension step at 72°C for 1.5 min, and concluded with a final extension step at 72°C for 5 min. The amplification products were checked by electrophoresis through 1.0% agarose gel, and then purified before DNA sequence analysis using a QIAquick PCR Purification Kit (QIAGEN, Korea) or Gel Purification Kit (QIAGEN, Korea) according to the manufacturer's instructions. Purified PCR products were then sequenced at Sangon Biotech (Shanghai) Co. Ltd. (http://www.sangon.com/).

Sequence analysis

The sequencing results obtained from Sangon Biotech Co. Ltd. Were edited and assembled by the software DNAMAN version 6.0 (Lynnon Biosoft Co., USA, www.lynon.com). Analogue of our sequences and nucleotide sequence comparisons were detected with Basic Local Alignment Search Tool (BLAST) network services against databases (http://www.ncbi.nlm.nih.gov/). The phylogenetic relationship among these pathogen species was analyzed based on the multiple sequence alignment of ITS1-5.8S-ITS2 region using DNAMAN version 6.0 software. Assembled sequences were deposited in the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). The NCBI GenBank accession numbers of ITS sequences from these pathogen species were shown in Table 1.

RESULTS AND DISCUSSION

Twenty-six healthy hot pepper leaves were sampled from seedlings grown in different geographical regions distributed in Chungbuk, Chungnam, Gyeongbuk, and Gangwon provinces of South Korea in July-August, 2016. Despite these pepper seedlings seemed healthy and strong, this could not prevent them from the pathogen infection. In addition, the advanced detection for pathogens was important for potential disease control.

The nrDNA ITS universal primer pairs, ITS1 and ITS4 (White *et al*., 20) were used in this present study. The efficient PCR amplification illustrated the universal primer pairs had good primer universality for pepper. PCR amplification of the nrDNA ITS region produced an approximately 558-bp fragment. No amplicon was observed in PCR tubes containing DNAs of CBB (No. 9 material), and CGS (No. 14 material).

The sequencing results were blasted in the BLAST services in NCBI server. All sequences showed that they had high identity rate with *Colletotrichum* species. Taken No. 1 material as example, its nrDNA ITS sequencing result showed 99% identity with *Colletotrichum* spp. QZ7L (LT632569), *C. acutatum* isolate ACUBP1 (KJ627843), *Glomerella acutata* isolate Mj6 (DQ454010), *C. scovillei* strain

Table 1. The detailed information of materials investigated in this study, and the accession numbers of the ITS nrDNA region genes logged in NCBI GenBank database.

Material No.	Host	Geographical distribution (GD)	GD Abb.	Specimen voucher	Fungi species	NCBI Acc. No.
1	*Capsicum annuum*	Chungbuk Chungju Salmi	CCS	Rbf-1	*Colletotrichum acutatum*	MF629897
2	*Capsicum annuum*	Chungbuk Jecheon Deoksa	CJD 1	Rbf-2	*Colletotrichum scovillei*	MF629898
3	*Capsicum annuum*	Chungbuk Danyang Yongchun	CDY	Rbf-3	*Colletotrichum scovillei*	MF629899
4	*Capsicum annuum*	Chungbuk Jecheon Bongya	CJB 1	Rbf-4	*Colletotrichum scovillei*	MF629900
5	*Capsicum annuum*	Chungbuk Jecheon Baekun	CJB 2	Rbf-5	*Colletotrichum scovillei*	MF629901
6	*Capsicum annuum*	Chungbuk Jeungpyeong Doan	CJD 2	Rbf-6	*Colletotrichum scovillei*	MF629902
7	*Capsicum annuum*	Chungbuk Yeongdong Yongsan	CYY	Rbf-7	*Colletotrichum scovillei*	MF629903
8	*Capsicum annuum*	Chungbuk Okcheon Cheongsan	COC	Rbf-8	*Colletotrichum acutatum*	MF629904
9	*Capsicum annuum*	Chungbuk Boeun Boeun	CBB	Rbf-9		
10	*Capsicum annuum*	Chungbuk Boeun Jangan	CBJ	Rbf-10	*Colletotrichum scovillei*	MF629905
11	*Capsicum annuum*	Chungnam Tean Nam	CTN	Rbf-11	*Colletotrichum scovillei*	MF629906
12	*Capsicum annuum*	Chungbuk Eumseong Soi	CES	Rbf-12	*Colletotrichum gloeosporioides*	MF629907
13	*Capsicum annuum*	Chungbuk Jincheon Deoksa	CJD 3	Rbf-13	*Colletotrichum scovillei*	MF629908
14	*Capsicum annuum*	Chungbuk Goesan Cheongcheon	CGC	Rbf-14		
15	*Capsicum annuum*	Chungbuk Goesan Sari	CGS	Rbf-15	*Colletotrichum acutatum*	MF629909
16	*Capsicum annuum*	Gyeongbuk Yecheon Gatcheon	GYG 1	Rbf-16	*Colletotrichum scovillei*	MF629910
17	*Capsicum annuum*	Gyeongbuk Cheongsong Hyeondong	GCH	Rbf-17	*Colletotrichum scovillei*	MF629911
18	*Capsicum annuum*	Gyeongbuk Yongyang Gagok	GYG 2	Rbf-18	*Colletotrichum scovillei*	MF629912
19	*Capsicum annuum*	Gyeongbuk Uiseong Danchon	GUD	Rbf-19	*Colletotrichum acutatum*	MF629913
20	*Capsicum annuum*	Gyeongbuk Andong Namseon	GAN	Rbf-20	*Colletotrichum scovillei*	MF629914
21	*Capsicum annuum*	University of Chungbuk Farm	UCF	Rbf-21	*Colletotrichum nymphaeae*	MF629915
22	*Capsicum annuum*	Gyeongbuk Cheongsong Pacheon	GCP	Rbf-22	*Colletotrichum acutatum*	MF629916
23	*Capsicum annuum*	Gyeongbuk Cheongsong Agricultral Center	GCAC	Rbf-23	*Colletotrichum simmondsii*	MF629917
24	*Capsicum annuum*	Gangwon Yeongwol	GY	Rbf-24	*Colletotrichum scovillei*	MF629918
25	*Capsicum annuum*	Gangwon Hoengseong	GH	Rbf-25	*Colletotrichum nymphaeae*	MF629919
26	*Capsicum annuum*	Gangwon Chuncheon	GC	Rbf-26	*Colletotrichum acutatum*	MF629920

P-11 (MG518626), *C. nymphaeae* isolate CDMF-1 (MF187553), and *C. simmondsii* isolate JA003 (KT844640). This result proved the validity and analogue of our sequencing results.

There were five anthracnose *Colletotrichum* species, including *C. acutatum*, *C. scovillei*, *C. gloeosporioides*, *C. nymphaeae*, and *C. simmondsii*, found from 26 healthy pepper materials. Among them, 14 pepper materials were tested to carry *C. scovillei* (accounting for 58.33%), 6 ones were tested to carry *C. acutatum* (accounting for 25.00%), 2 to carry *C. nymphaeae* (accounting for 8.33%), and 1 to carry *C. gloeosporioides* (accounting for 4.17%) and *C. simmondsii* (accounting for 4.17%), respectively (Table 1). No amplicon obtained from CBB and CGS materials suggested that these both pepper materials had not been infected by any pathogen.

As known, *C. acutatum* is still considered as one of the most destructive and the most common anthracnose pathogens worldwide (Wharton and Dieguez-Uribeondo, 19). In this study, 25% of pepper materials carried this pathogen *C. acutatum*,

however, most pepper materials carried *C. scovillei*, which belongs to the *C. acutatum* species complex (Gao *et al*., 10). *C. scovillei* was found to cause severe fruit rot of sweet pepper (Caires *et al*., 3), and this disease caused large loss in production in Japan from 2005 to 2011. *C. gloeosporioide*s was found to induce anthracnose of chilli in the Philippines (Than *et al*., 16), there were few reports of the pepper infection in other regions. The other both anthracnose pathogens, *C. nymphaeae* and *C. simmondsii* had been reported to infect fruits, including grapevine (*Vitis vinifera* cv. Red Globe) (Liu *et al*., 14), peach (Chen *et al*., 5), strawberry (Weber *et al*., 18), vegetables, including celery (Fujinaga *et al*., 9), and other plants, including water-lilies (Johnson *et al*., 13), safflower (Vichová *et al*., 17), and caused large loss in crop production, but there had been no report about the infection evidence in peppers. Thus, this study is the first time to find the infection capability of *C. nymphaeae* and *C. simmondsii* in peppers.

To check the genetic diversity among these pathogens, the phylogenetic relationship was analyzed using the DNAMAN software 6.0 version. Seen from the phylogenetic tree, it suggested that *C. nymphaeae* showed the farthest relationship with other pathogen species, locating the outermost clade (Fig. 1). Because *C. scovillei* is *C. acutatum* species complex (Gao *et al*., 10), these both pathogen species showed relatively nearer phylogenetic relationship: most *C. acutatum* and *C. scovillei* materials were separated into a large clade sharing 98% identity rate with other species, except GUD (belonging to *C. acutatum*, No. 19 material). GUD (No. 19 material) located in the outside of the large clade, forming a clade with an independent material. *C. gloeosporioides* and *C. simmondsii* had one material, respectively, and they formed an independent clade, respectively (Fig. 1).

Searching the nrDNA ITS sequence reports of *Colletotrichum* species in the NCBI GenBank database, we collected 63 existing sequences including 45 different *Colletotrichum* species and 3 unknown/ unclear *Colletotrichum* species. Combined with our 24 sequences, we constructed the phylogenetic tree of these 87 *Colletotrichum* species based on the observed divergency (Fig. 2). Although this genus had similar biological characteristics, there was rich genetic diversity occurring in the nrDNA ITS region sequence. All *C. scovillei* and *C. acutatum* materials were divided into one clade, sharing the lowest 90% identity rate with each other. This grouping pattern was the same as the analysis result only using our 24 materials investigated in this study (Fig. 1). Among all *C. scovillei* and *C. acutatum* materials, CJB2 (No. 5 material) and GUD (No. 19 material) were considered as the outermost one of *C. scovillei* and *C. acutatum*, respectively. All 6 *C. gloeosporioides* materials were divided into one clade, sharing the lowest 97% identity rate with each other. *C. brevisporum*, *C. lobatum*, *C. magnum*, and other 17 species were divided into one clade. The existing 5 *C. nymphaeae* and 2 *C. simmondsii* materials were divided into the *C. acutatum* clade, while our 2 *C. nymphaeae* and 1 *C. simmondsii* materials showed the farthest phylogenetic relationship with them (Fig. 2). Seen from the nucleotide sequence, the genetic variation mainly appeared in the ITS2 region, that induced the grouping difference.

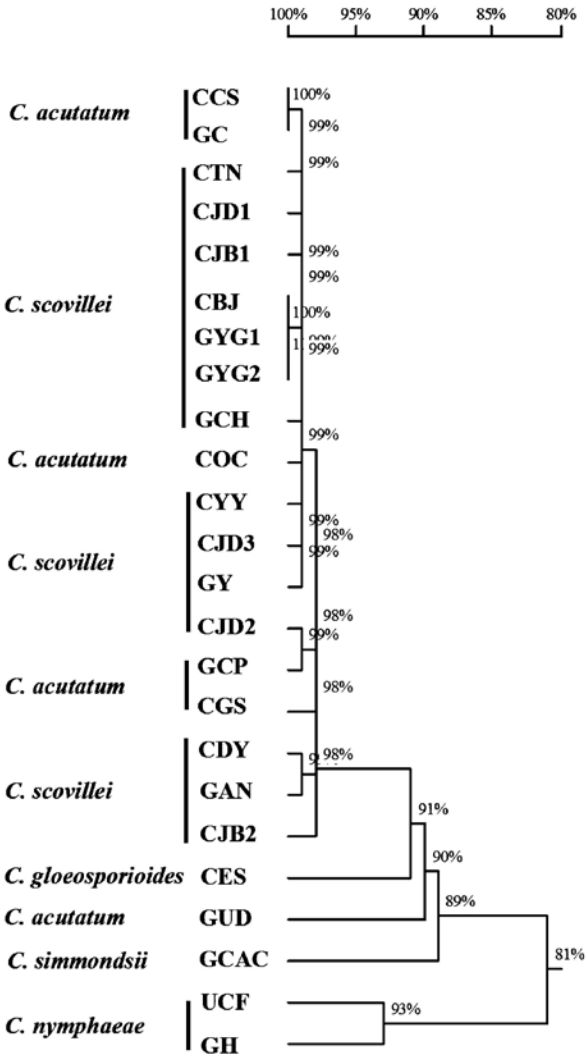

Fig. 1. Phylogenetic relationship among 24 *Colletotrichum* pathogens investigated in this study.

Sequence analysis result indicated that there was rich genetic variation among the *Colletotrichum* species, and even within one specific *Colletotrichum*

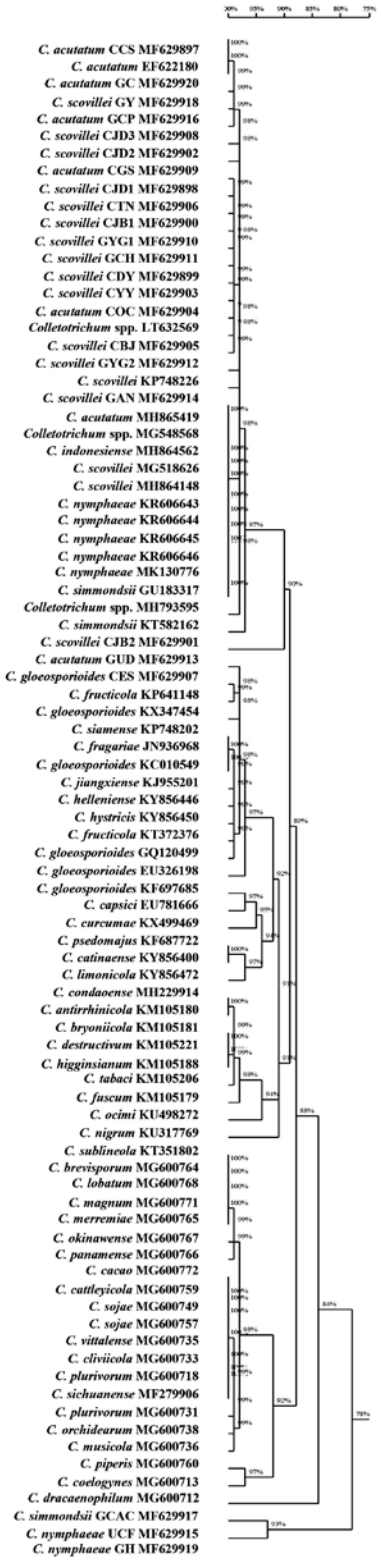

Fig. 2. Phylogenetic relationship among 87 *Colletotrichum* materials, including 45 different species, 3 unknown/ unclear *Colletotrichum* species, and our samples investigated in this study.

species. This work also suggested that except the common pepper anthracnose pathogens *C. acutatum*, *C. scovillei*, and *C. gloeosporioides*, *C. nymphaeae* and *C. simmondsii* also were firstly found to be able to infect peppers in South Korea. Through sequence analysis result, we found that our *C. nymphaeae* and *C. simmondsii* materials had relatively farthest phylogenetic relationship with other *Colletotrichum* species. Whether this is the main reason of that these both species was firstly found in peppers should be further studied.

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