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PCR Based Assays for the Specific Detection of *Colletotrichum* species Causing Apple Bitter Rot in Korea

Abdulkareem Abdullahi, Heung Tae Kim*
Department of Plant Medicine. College of Agriculture, Life and Environmental Sciences,
Chungbuk National University, Korea

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Abstract *Colletotrichum* species of the *C. gloeosporioides* species complex are the major pathogens of bitter rot of apples in Korea. To ensure early and quick detection of these species, species-specific primers based on the single nucleotide polymorphisms (SNPs) in the nucleotide sequences of the β-tubulin gene were designed and used in PCR assays. Primers were designed by making the SNP the 3‘ end and introducing a single nucleotide artificial mismatch at the penultimate base to the SNP. Primer pairs Cae-Bt462F/CgSc-Bt691R, Cfr-Bt492F/CgSc-Bt691R, Cgl-591F/CgSc-Bt691R and Csi-Bt567F/CgSc-Bt691R successfully amplified specific PCR fragments from *C. aenigma, C. fructicola, C. gloeosporioides* and *C. siamense* respectively in pure cultures and inoculated apple fruits. Each primer pair only amplified fragments from their respective species. The sensitivity assay to determine the lowest detectable DNA concentration of the primers showed that the primers could detect as low as 10 to 100 pg of purified DNA. A duplex PCR assay was conducted with primer pairs Cfr-Bt492F/CgSc-Bt691R and Csi-Bt567F/CgSc-Bt691R for the detection of *C. fructicola and C. siamense*, the two most isolated species from bitter rot infected apple in Korea. The primers could specifically detect each species from one reaction. This PCR-based protocol could be used for quick detection of *Colletotrichum gloeosporioides* species complex causing apple bitter rot in Korea and could save the cost of bitter rot diagnosis.

Keywords: Apple bitter rot, PCR, *Colletotrichum* species, Species-specific primer, Duplex PCR

Introduction

Colletotrichum species within the *C. gloeosporioides* and *C. acutatum* species complexes have been reported to cause bitter rot of apples in Korea. Among these species, *C. fructicola, C. siamense, C. gloeosporioides* and *C. aenigma,* within the *C. gloeosporioides* species complex and *C. fiorinieae* and *C. nymphaeae*, belonging to the *C. acutatum* species complex have over the years been isolated from bitter rot-infected apples in Korea (Kim et al., 2018; Lee et al., 2007; Lee et al., 2021; Oo et al., 2018; Park et al., 2018). Within the last three years, most of the isolates from bitter rot-infected apples have been *C. fructicola* and *C. siamense* (Kim et al., 2023). Interestingly, the two species may differ in their sensitivities to the commonly used fungicides, particularly those of the benzimidazoles and quinone outside inhibitors (Abdullahi et al., 2023). Therefore, identification of these species is necessary to understand disease epidemiology and develop strategies to control the disease successfully (Cai et al., 2009). Current identification of these species relies mostly on the use of some genetic markers, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-tubulin (TUB2), actin (ACT), calmodulin (CAL), chitin synthase (CHS-1), histone-3 (His3) and internal transcribed spacer (ITS) region (Liu et al., 2012; Vieira et al., 2020). Owing to their high sequence similarity, one marker is insufficient and at least three of these markers offer sufficient information for discrimination of these species (Vieira et al., 2020). The high cost of sequencing these genes and the time spent in preparing samples pose challenges to the quick identification of these species, particularly for small laboratories.

Species-specific polymerase chain reaction (PCR) based assays are commonly used for the identification and detection of fungal species, offering less time and saving cost of gene sequencing. Species-specific primer designs to target unique regions of the target species with high sequence variability have been used for the identification of some fungal organisms (Ismadi et al., 2023; Luo and Mitchell,

*Corresponding author
E-mail: htkim@chungbuk.ac.kr

2002; Yamashita et al., 2018). However, higher consensus regions among the species within *C. gloeosporioides* species complex in the commonly used genetic markers have made this primer design difficult. ITS region for example is only ideal for designing specific primers to detect fungi belonging to different genera, due to its variability among these genera but highly limited in specific primer design for fungi within a species complex (White et al., 1990). Though GAPDH, His3, and TUB2 have been adjudged three of the best markers for discriminating members of this complex (Vieira et al., 2020), most of the sequence variability found among these species in GAPDH and His3 including ACT are contained in the intron region of the gene. TUB2 contains more variable sequences among these species in the exon region of the gene but are mainly single nucleotide polymorphisms (SNPs).

Several studies have reported the use of SNPs in species-specific primer design for the identification of some fungal species and other microorganisms (Kalendar et al., 2022; Liu et al. 2012; Nawaz et al., 2018). The 3' end of the primer (forward and/or reverse) is usually targeted to coincide with the SNP. However, a single base mismatch at the very 3' end still allows amplification, though at a reduced efficiency for some cases. The consensus is that a two-base mismatch at the 3' end generally prevents amplification (Ye et al., 2012). It is difficult to find a two-base mismatch in the nucleotide sequences of *Colletotrichum* species within the *C. gloeosporioides* species complex infecting apples in the commonly used genetic markers. With the limitation of using only SNPs for designing species-specific primers, and the need for quick detection of these species directly from bitter rot-infected apple fruits, this study was

Table 1. *Colletotrichum* species isolates used in this study

No.	Isolates	*Colletotrichum* species	Source	Year of isolation	NCBI accession number
1	16MPDY5	*C. aenigma*	Chungbuk, Korea	2016	PP704640
2	16MPDY19	*C. aenigma*	Chungbuk, Korea	2016	PP704641
3	JlJaCG22-2-1	*C. aenigma*	Jeonbuk, Korea	2022	PP704642
4	YC2RUB3	*C. aenigma*	Gyeongbuk, Korea	2021	PP704643
5	MPYD10	*C. fructicola*	Chungbuk, Korea	2016	PP704644
6	MPYD11	*C. fructicola*	Chungbuk, Korea	2016	PP704645
7	DO35	*C. fructicola*	Gyeongbuk, Korea	2015	PP704646
8	DO39	*C. fructicola*	Gyeongbuk, Korea	2015	PP704647
9	16MPDY1	*C. gloeosporioides*	Chungbuk, Korea	2016	PP704648
10	16MPDY4	*C. gloeosporioides*	Chungbuk, Korea	2016	PP704649
11	16MPDY16	*C. gloeosporioides*	Chungbuk, Korea	2016	PP704650
12	GgYcCG23-3-1	*C. gloeosporioides*	Gyeonggi, Korea	2023	PP704651
13	16MPYD19	*C. siamense*	Chungbuk, Korea	2016	PP704652
14	16MPDY2	*C. siamense*	Chungbuk, Korea	2016	PP704653
15	16MPDY3	*C. siamense*	Chungbuk, Korea	2016	PP704654
16	16MPBH1	*C. siamense*	Gyeongbuk, Korea	2016	PP704655
17	16MPNJ1	*C. fioriniae*	Jeonnam, Korea	2016	PP471126
18	16MPBE3	*C. fioriniae*	Chungbuk, Korea	2016	PP471127
19	15MPYS4	*C. fioriniae*	Chungnam, Korea	2015	PP471129
20	DO5	*C. fioriniae*	Gyeongbuk, Korea	2015	PP471130
21	16MPBE1	*C. nymphaeae*	Chungbuk, Korea	2016	PP471132
22	16MPBE6	*C. nymphaeae*	Chungbuk, Korea	2016	PP471133
23	APTW13	*C. nymphaeae*	Gyeongbuk, Korea	2015	PP471134
24	APTW17	*C. nymphaeae*	Gyeongbuk, Korea	2015	PP471135

aimed at developing an SNP-based species-specific PCR assays for the detection of *Colletotrichum* species within the *C. gloeosporioides* species complex causing bitter rot of apples in Korea.

Materials and Methods

DNA extraction

Twenty-four isolates of *Colletotrichum* species were used in this study (Table 1). These included four isolates each of *C. aenigma, C. fructicola, C. gloeosporioides*, and *C. siamense*, all belonging to *C. gloeosporioides* species complex and four isolates each of, which are *C. fioriniae* and *C. nymphaeae* of the *C. acutatum* species complex. These species were isolated from bitter rot-infected apples in 2015, 2016, 2021, 2022, and 2023. These isolates, previously stored at -70℃ in 20% glycerol were revived on potato dextrose agar (PDA; Difco™, Becton, Dickinson and Company, Sparks, MD21152 USA). After incubation in the dark at 25℃ on PDA, the mycelia were harvested and transferred into a 2.0 mL microcentrifuge tube, freeze-dried for 24 hours, and used for DNA extraction. Total DNA from each isolate was extracted using a Plant SV mini extraction kit (GeneAll Biotechnology, Korea) following the manufacturer's instructions.

Species−specific primer design

The full length sequences of the TUB2 region of these isolates were amplified using three primer pairs (Table 2). The amplicons were purified and sequenced using Sanger sequencing. The obtained sequences of these isolates together with other reference sequences of the *Colletotrichum* species were analysed using the CLUSTAL W program of MEGA 11 software. From the aligned sequences, SNPs for each species were identified. Species-specific forward primers were designed with the SNP at the 3' end while altering the penultimate base of the primer. For instance, in designing a specific primer for *C. aenigma*, an SNP with thymine (T) was identified at the 480th mRNA nucleotide position of the TUB2, whereas all other *Colletotrichum* species carry a cytosine (C) at the same position. The 479th base which was also a C was then substituted with another T to create a mismatch at the penultimate base end of the primer. This procedure creates only one mismatch from the target template, but two mismatches at the 3'-end of the non-target template, thus avoiding possible amplification of the latter. A 19-base primer was then designed from the 480th base backward for detection of *C. aenigma*. This procedure was followed for other *Colletotrichum* species. A common reverse primer from the conserved region of the gene was designed for all the species. The designed primers were analysed for GC content, Tm, as well as hairpin, self-dimer, and heterodimer formation using integrated DNA technologies online oligo analyser tools (https://sg.idtdna.com/calc/analyzer). SNPs that would result in a primer with more than 70% GC content and more than three 'G's or 'C's in the 3'-end due to the creation of a C or G mismatch were avoided. This is because of the possibility of potential annealing at multiple sites. Where this was not possible as in the case of *C. fructicola*, another mismatch with a T was introduced towards the 5' end of the primer.

Validating primers for specificity and sensitivity

DNA templates of four representative isolates of each species were selected to test the specificity of the primers. For each primer pair, PCR was carried out in a 20 μL reaction volume containing 4 μL master mix (EzPCR 5X PCR master mix, Elpis Biotech.), 1 μL each of the forward and reverse primers, 2 μL gDNA and 12 μL sterile distilled water (SDW). To determine the lowest detectable DNA template concentration of the species-specific primers, DNA templates of the species were serially diluted from 100 ng/μL to 10 pg/μL, and 1μL was used as a template for the PCR. PCR conditions included initial denaturation at 95℃ for 2 mins, followed by 30 cycles

Table 2. Primers used to amplify full length of β-tubulin gene sequences

Primers	Sequence (5→3)	Direction	Reference
T1	AACATGCGTGAGATTGTAAGT	F	O'Donnell and Cigelnik (1997)
BTUB4RD	CCRGAYTGRCCRAARACRAAGTTGTC	R	Woudenberg et al. (2009)
BTUB2FD	GTBCACCTYCARACCGGYCARTG	F	Woudenberg et al. (2009)
TB2R	TGAGCTCAGGAACRCTGACG	R	Peres et al. (2004)
TB2L	GYTTCCAGATYACCCACTCC	F	Peres et al. (2004)
TB2-4R	CTAAGAGAGGTATATCTAGAAGCCC	R	This study

each involving denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, and then final extension at 72°C for 5 mins. The PCR products were separated by electrophoresis in 1% agarose gel in Tris-Borate (TBE) buffer for 40 min at 100 volts and viewed using a transilluminator.

Detection of *Colletotrichum* species from inoculated apple fruits

Eight representative isolates comprising *C. aenigma, C. fructicola, C. gloeosporioides*, and *C. siamense* were selected for inoculation into apple fruits. Healthy apple fruits were collected from a commercial orchard, washed with tap water, and disinfected in 1% sodium hypochlorite (NaOCl) for three minutes. The disinfected apples were washed with sterilized water three times, dried in a laminar flow hood, and then placed on a sterilized plastic saucer in a sterilized plastic box with wet tissue. The apples were inoculated with 10 μL each of 10^4 spore/mL concentration of the isolates. Control apple fruit received SDW. Three replications were maintained for each isolate as well as the control. Plastic boxes containing inoculated apple fruits and the control placed on moist paper towels were sealed and incubated at 25°C for 72 hours in the dark to allow for a post-inoculation period of high humidity. Thereafter seals were removed and boxes were opened for air and tissue paper wetting. Ten days after inoculation, infected tissues were excised for DNA extraction using the method described earlier. The species-specific PCR assay and agarose gel electrophoresis were conducted as described above.

Duplex PCR assay

One isolate each of *C. fructicola* and *C. siamense* was selected for a multiplex PCR assay in a 20 μL PCR mixture containing 4 μL master mix (EzPCR 5X PCR master mix, Elpis Biotech.), 0.5 μL (0.25 pmol/μL) each of the forward primers, 1 μL (0.5 pmol/μL) of the reverse primer, 1 μL gDNA each of the isolate and 12 μL sterile distilled water (SDW). PCR conditions and agarose gel electrophoresis are described above.

RESULTS

Species−specific primer design

The sequence similarity in the beta-tubulin gene of *C. aenigma, C. fructicola, C. gloeosporioides*, and *C. siamense* associated with apple bitter rot was analysed. A comparison of the sequences indicated that the TUB2 genes of these species are highly conserved with only synonymous SNPs distinguishing among the species, which were either by transition (>90%) or by transversion (<10%). Using the SNPs with the method described above, four species-specific forward primers and a common reverse primer were designed to amplify the sequences of four *Colletotrichum* species causing bitter rot of apples (Table 3).

Primer specificity

The specificity of the primers was tested using genomic DNA extracted from the mycelia of the isolates (Table 2). The four primer pairs for members of the *C. gloeosporioides* species complex could amplify a fragment from the four species respectively. Primer pair Cae-Bt462F/CgSc-Bt691R produced 230 bp amplicon from *C. aenigma* isolates 16MPDY5, 16MPDY19, JlJaCG22-2-1, and YC2RUB3 and none from other isolates. The primer pair Cfr-Bt492F/CgSc-Bt691R produced 200 bp amplicon from *C. fructicola* isolates 16MPYD10, 16MPYD11, DO35, and DO39, but none from other isolates. 16MPDY1, 16MPDY4, 16MPDY16, and GgYcCG23-3-1 isolates of *C. gloeosporioides* were amplified by the primer pair Cgl-Bt591F/CgSc-Bt691R, producing 101 bp fragment but none of the isolates from other species was amplified. Primer pair Csi-Bt567F/CgSc-Bt691R produced 125 bp amplicon from the *C. siamense* isolates 16MPYD19, 16MPDY2, 16MPDY3, and 16MPBH1, but none from the other isolates (Fig. 1). The primers were also used to amplify *C. fioriniae* and *C. nymphaeae* of the *C.*

Table 3. *Colletotrichum* species-specific primers designed from *TUB2* gene

Species	Primer	Sequence (5' → 3'))	Direction	Product size (bp)
C. aenigma	Cae-Bt462F	GATCCGTGAGGAGTTCCTT	Forward	230
C. fructicola	Cfr-Bt492F	GGTCACCTTCTCCGTCGCC	Forward	200
C. gloeosporioides	Cgl-Bt591F	CGAGACCTTCTGCATTGTT	Forward	101
C. siamense	Csi-Bt567F	CCACCAGCTGGTCGAGATT	Forward	125
	CgSc-Bt691R	CAGAGACCAGGTGGTTCAG	Reverse	

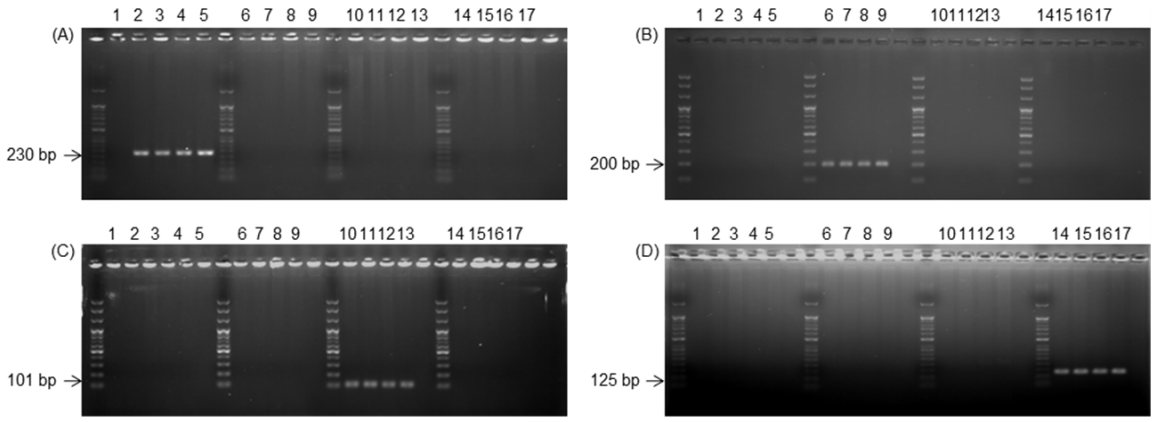

Fig. 1 Gel electrophoresis showing the specific bands of the fragment of β-tubulin gene produced by each primer. (A); *Colletotrichum aenigma* specific primers Cae-Bt462F/CgSc-Bt691R, (B); *C. fructicola* specific primers Cfr-Bt492F/CgSc-Bt691R, (C); *C. gloeosporioides* specific primers Cgl-591F/CgSc-Bt691R, (D); *C. siamense* specific primers Csi-Bt567F/CgSc-Bt691R. Four isolates of each of the four species of *Colletotrichum* were selected and used in the experiment. 1; negative control, 2-5; *C. aenigma* 16MPDY5, 16MPDY19, JlJaCG22-2-1, and YC2RUB3, 6-9; *C. fructicola* 16MPYD10, 16MPYD11, DO35, and DO39, 10-13; *C. gloeosporioides* 16MPDY1, 16MPDY4, 16MPDY16, and GgYcCG23-3-1, 14-17; *C. siamense* 16MPYD19, 16MPDY2, 16MPDY3, and 16MPBH1.

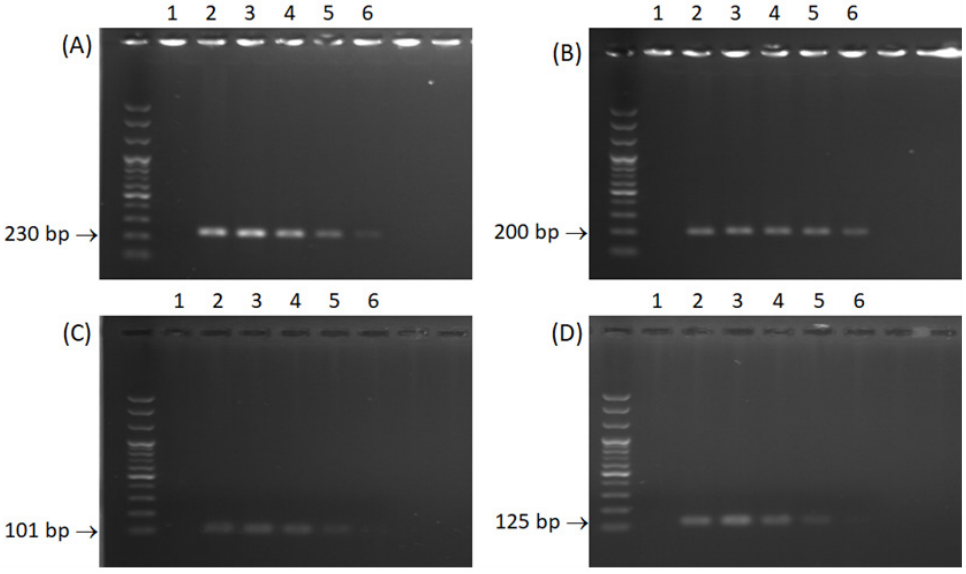

Fig. 2 Gel electrophoresis showing the specific bands of the fragment of β-tubulin gene produced by each primer for each DNA concentration. (A); *Colletotrichum aenigma* specific primers Cae-Bt462F/CgSc-Bt691R, (B); *C. fructicola* specific primers Cfr-Bt492F/CgSc-Bt691R, (C); *C. gloeosporioides* specific primers Cgl-591F/CgSc-Bt691R, (D); *C. siamense* specific primers Csi-Bt567F/CgSc-Bt691R. One isolate per *Colletotrichum* species was used in this experiment, *C. aenigma* 16MPDY5, *C. fructicola* 16MPYD10, *C. gloeosporioides* 16MPDY1, and *C. siamense* 16MPYD19. The numbers indicate the concentration of DNA used in this experiment. As follows: 1; negative control, 2; 100 ng/μL, 3; 10 ng/μL, 4; 1 ng/μL, 5; 100 pg/μL, and 6; 10 pg/μL.

acutatum species complex isolated from apple but no band was produced (data not shown).

Primer sensitivity

The sensitivities of the primers were tested by 10-fold dilution of template DNA of 100 ng/μL, 10 ng/μL, 1 ng/μL, 100 pg/μL and 10 pg/μL. Primer pair Cae-Bt462F/CgSc-Bt691R could detect DNA concentration as low as 10 pg/μL, while the lowest detectable DNA concentration by the three other primers was 100 pg/μL each (Fig. 2).

Detection of the *Colletotrichum* species from inoculated apple fruits

DNA from bitter rot lesions resulting from inoculated apple fruits was extracted and a PCR assay was carried out to determine whether the primers could detect *Colletotrichum* species in artificially inoculated apple fruits. The results showed that the primer pairs of Cae-Bt462F/

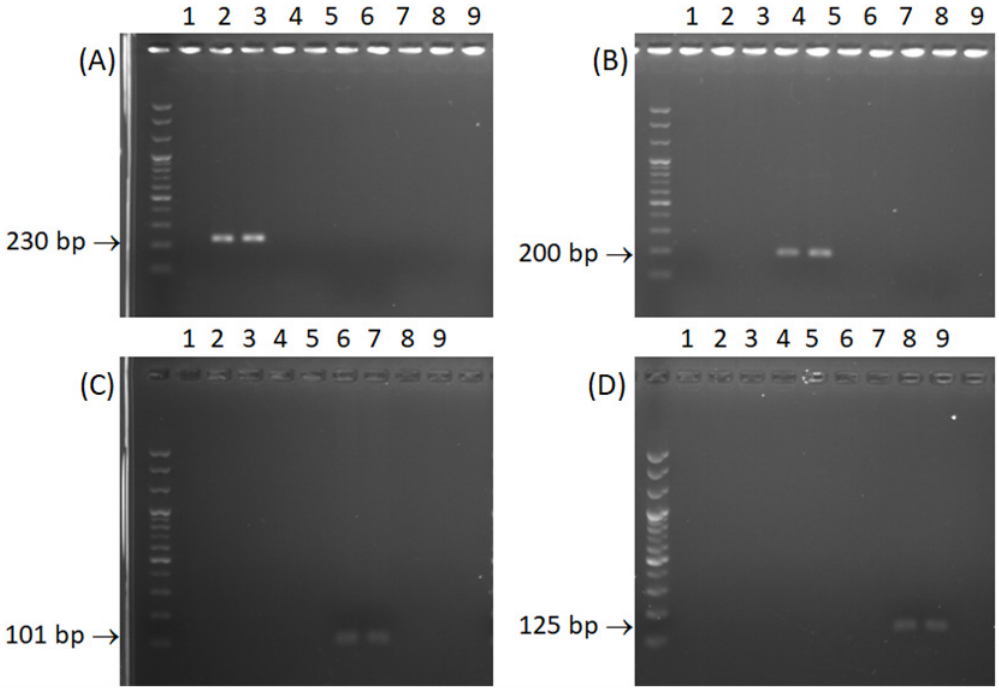

Fig. 3 Gel electrophoresis of PCR with each species-specific primer using gDNA extracted from diseased apples as a template. In this experiment, 4 primer sets as Cae-Bt462F/CgSc-Bt691R (A), Cfr-Bt492F/CgSc-Bt691R (B), Cgl-591F/CgSc-Bt691R (C) and Csi-Bt567F/CgSc-Bt691R (D) were used to detect *Colletotrichum* spp. included into *C. gloeosporioides* species complex. For the inoculation of *Colletotrichum* spp. to apple fruits, conidial density was adjusted to 1×10^6 conidia/mL. After inoculation, apple fruits were kept at 25℃ for 3 days in humidity chamber. Thereafter, gDNA was extracted from the initial lesions 7 days after inoculation. Numbers indicate the isolates of *Colletotrichum* species used in the experiment, where two isolates of each *Colletotrichum* species were used, as follows. 1; negative control, 2; *Colletotrichum aenigma* 16MPDY5, 3; *C. aenigma* JlJaCG22-2-1, 4; *C. fructicola* 16MPYD10, 5; *C. fructicola* DO39, 6; *C. gloeosporioides* 16MPDY1, 7; *C. gloeosporioides* GgYcCG23-3-1, 8; *C. siamense* 16MPYD19, and 9; *C. siamense* 16MPDY2.

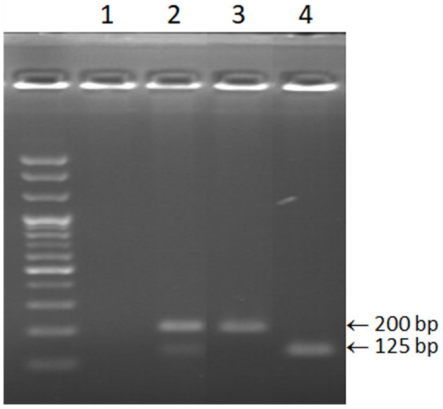

Fig. 4 Gel electrophoresis showing duplex PCR for the detection of *Colletotrichun fructicola* and *C. siamense* amplified with primer pairs Cfr-Bt492F/CgSc-Bt691R and Csi-Bt567F/CgSc-Bt691R, respectively. 1; negative control, 2; *C. fructicola* 16MPYD10 and *C. siamense* 16MPDY2, 3; *C. fructicola* 16MPYD10, 4; *C. siamense* 16MPDY2.

CgSc-Bt691R, Cfr-Bt492F/CgSc-Bt691R, Cgl-Bt591F/CgSc-Bt691R, and Csi-Bt567F/CgSc-Bt691R yielded the expected single band of 230 bp, 200 bp, 101 bp, and 125 bp respectively (Fig. 3).

Duplex PCR Assay

A duplex PCR assay with all four primer pairs could not produce distinct bands because of the closeness of the band sizes, 101bp/125bp and 200bp/230bp for *C. gloeosporioides*/*C. siamense* and *C. fructicola*/*C. aenigma* respectively. A duplex PCR assay involving primer pairs Cfr-Bt492F/CgSc-Bt691R and Csi-Bt567F/CgSc-Bt691R successfully detected *C. fructicola* and *C. siamense* from one reaction, producing 200 bp and 125 bp respectively (Fig. 4).

Discussion

Apple bitter rot caused by *Colletotrichum* species is one of the most devastating fruit diseases in Korea. At least six *Colletotrichum* species belonging to *C. gloeosporioides* and *C. acutatum* complexes have been identified over the years to be associated with this disease (Kim et al., 2018; Lee et al., 2007; Oo et al., 2018; Park et al., 2018). However, the current status of the disease in the country shows that members of the *C. gloeosporioides* complex are the major pathogens of apple bitter rot (Abdullahi et al., 2023). Rapid identification

of these species is necessary for epidemiology and management strategies. In this study, we designed single nucleotide polymorphism (SNP) based primers for the specific detection of these species from pure culture and disease apple fruits. The use of SNP for species-specific primer design for fungal species identification has been demonstrated in numerous studies, particularly for species with high sequence similarity in their genome (Liu et al., 2012; Nawaz et al., 2018; Xu et al., 2016). The most common SNP-based primer design places the SNP at the 3' end of the primer (Liu et al., 2012), though there is a report of the SNP at the 5' end of the forward primer (Nawaz et al., 2018). The major challenge of the SNP-based species-specific primer design is the possibility of amplification of the non-targets but a two-base mismatch at the 3' end generally prevents amplification according to Ye et al. (2012). Xu et al. (2016) reported that introducing mismatches at the 2nd, 3rd, or 4th base from the SNP increases the specificity of the primers but with varying degrees and concluded that mismatches at the 3rd base from the SNP were the most efficient in discriminating the two lines of the *Brassica oleracea*. Attempts to design SNP-based primers with mismatches specified in that study could not produce the desired results. Here we introduced a mismatch of the same base as the SNP of the target at the 1st base (penultimate base) from the SNP, based on the nucleotide sequence of the β-tubulin gene. Using this method, species-specific primers designed with thymine as the SNP of the target had the highest efficiency and were more specific than those with cytosine or guanine. Those with a double G or C at the 3' end, as in the case of the forward primer for detection of *C. fructicola* produced multiple bands (data not shown), which was probably due to high G/C content and/or more than three 'G's or 'C's in the 3'-end as a result of creation of a C or G mismatch (Javed and Ebertz, 2022). This was resolved by the creation of another thymine mismatch towards the 5' end (16th base from SNP) of the primer.

McHenry and Acimovic (2024) reported species-specific real-time PCR assays for the detection of *Colletotrichum* species causing bitter rot of apples using primers designed from some gene regions specific for each species, but the study did not report direct detections from diseased apple fruits. The four primers designed in this study could detect each species from pure culture and diseased apple fruits, enhancing quicker detection of each species. The primers were also tested for other *Colletotrichum* species belonging to the *C. acutatum* species complex and none of these species could be detected by these primers. The sensitivity of a primer is important for early diagnosis of disease plants (Xu et al., 2016). Though the primers could detect as low as 100 pg DNA of pure culture of their respective species, the multiplex PCR assay involving the four primer sets could not successfully discriminate the four species. However, *C. fructicola* and *C. siamense*, the two most isolated species from bitter rot in Korea were successfully discriminated by a duplex PCR. The response of these two species to the commonly used fungicides differ markedly (Abdullahi et al., 2023), thus, quick detection could improve the fungicide management strategy of apple bitter rot in Korea. Also, this PCR protocol is much easier than the morphological method and more cost-effective than gene sequencing. In addition, the unique primer design approach described in this study could also be used to design species specific primers for other fungal species.

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Author Information and Contributions

Abdulkareem Abdullahi, Department of Plant Medicine, College of Agriculture, Life and Environment Science, Chungbuk National University, PhD student, Research investigation, development of allele-specific PCR, data analysis and first draft preparation. https://orcid.org/0000-0002-3841-2356

Heung Tae Kim, Department of Plant Medicine, College of Agriculture, Life and Environment Science, Chungbuk National University, Professor, Establishment of experimental plan and methodology proposal, writing original paper and editing.
https://orcid.org/0000-0001-7132-0587

Conflict of Interest

The authors declare that they have no conflict of interest.

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