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Development of Rapid Molecular Detection Marker for *Colletotrichum* spp. in Leaf and Fruit Tissues of Sweet Persimmon

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Abstract Sweet persimmon (*Diospyros kaki* Thunb.) is widely cultivated in the southern part of Korea and its cultivation is increasing. However, anthracnose disease caused by *Colletotrichum* species is one of the major hinderances to the cultivation and production of sweet persimmon. Therefore, in the current study, PCR was used to specifically detect *Colletotrichum* spp., based on the sequences of the ITS II regions in the rDNA. Using the sequence data, CO-1 was designated to detect *Colletotrichum* together the with ITS 4 primer. The result showed that a single segment of ca. 500 bp was observed only in *Colletotrichum*, but not in any other fungal and bacterial isolates. The annealing temperatures and template DNA quantites were also investigated to identify optimal conditions for detection. Using these species-specific primers, a unique band was obtained at annealing temperatures ranging from 55°C and 61°C and template DNA levels from 10 pg– 10 μg.

Key words: Anthracnose, *Colletotrichum* spp., ITS region, molecular detection, rDNA, sweet persimmon

The genus *Colletotrichum* includes some of the most economically important fungi and destructive plant pathogens. The most common form of *Colletotrichum* diseases are anthracnoses, which are very similar, if not identical, to the diseases caused by *Glomerella* [1]. As such, the latter would appear to be the sexual stage of most or all species of *Colletotrichum* (*Gloeosporium*). The taxonomy of *Colletotrichum* species is based on its pathogenicity and features, such as a conidial shape and size, septae, and appressorial morphology. However, since morphological characteristics can vary with culture conditions and overlap of phenotypes, these criteria are not always reliable [2, 3, 5, 9]. Therefore, DNA sequence comparisons have been used to examine a number of *Colletotrichum* species, and the sequences of the internal transcribed spacer (ITS) regions of the ribosomal DNA have proven to be particularly useful in delineating members of this genus [10, 15, 16, 17, 18, 19].

In recent years, many researchers have studied the ITS regions of ribosomal DNA to analyze genetic differences and taxonomical relationship among *Colletotrichum* species [8, 10, 13, 19]. The ITS regions, noncoding and varible, and 5.8S rRNA gene, coding and conserved, are useful in measuring phylogenetic relationships among closely related fungi [4, 7, 11, 21]. Since ribosomal regions evolve in a concerted fashion, they reveal a low intraspecific polymorphism and high interspecific variability [11], which has proven to be very useful in the identification of *Colletotrichum* spp. [14, 15, 16]. If *Colletotrichum* spp., which can severely decrease the yield of sweet persimmons, could be rapidly and accurately detected, this would be useful in differentiation of *Colletotrichum* spp. from other *Colletotrichum* species, prediction and control of anthracnoses, and reduction of pesticide use, resulting in the harvest of healthy sweet persimmon. Accordingly, the current work describes the development of a PCR primer derived from the ITS regions of rDNA repeats for the specific detection of *Colletotrichum* spp. in sweet persimmons. The specificity and absence of cross reactivity were tested by using *Colletotrichum* spp. and representatives of other fungal genera.

The *Colletotrichum* spp. isolates used in the study were either isolates from sweet persimmons or other researchers (Table 1). The ITS II regions between 5.8S and 28S were amplified for all *Colletotrichum* spp. with the ITS 3 (5'-GCATCGGATGAACGCAGC-3') and ITS 4 (5'-TCCTC-CGCTTATTGATATGC-3') primers (Fig. 1A). A single band of ca. 627 bp was obtained from all *Colletotrichum* spp. isolated from Kyungju, Kimhae, Changwon, and

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Table 1. Isolates of *Colletotrichum* spp. used in current study.

Isolate no.	Isolate name
1	Kyungju 1
2	Kyungju 2
3	Kyungju 3
4	Kyungju 5
5	Kimhae 3
6	Kimhae 10
7	Changnyung 17
8	Kimhae 11
9	Kimhae 27
10	Milyang 18
11	Milyang 19
12	Milyang 20
13	Changwon 3
14	Changwon 15
15	Changwon 29
16	Milyang 11
17	Kimhae 22
18	Kyungju 6
19	Changwon 4
20	Changwon 8
21	Changwon 19
22	Milyang 7
23	Changwon 27
24	Changnyung 16
25	Changnyung 18

Changnyung (Fig. 1B). The PCR product from the Kyungju 3 isolate was used for analyzing the nucleotide sequences. As shown in Fig. 2, the single band of Kyungju

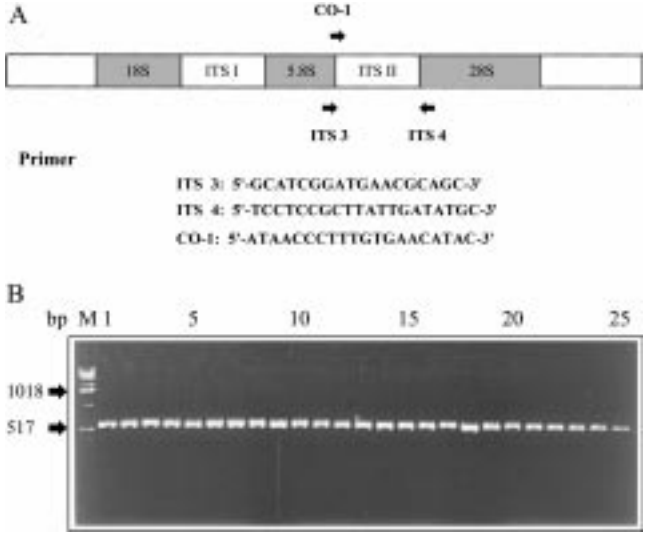

Fig. 1. A. Genetic map of a portion of the rDNA repeat showing the location of the oligonucleotide primer site used to amplify rDNAs from *Colletotrichum* spp. B. PCR amplified portion of ITS II region in *Colletotrichum* spp. The numbers on top of the lane indicate the isolate numbers in Table 1.

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  1 TCCTCCGCTT ATTGATATGC TTAAGTTCAG CGGGTATTCC
 41 TACCTGATCC GAGGTCAACC TGTAAAGAAT TTGGGGGTTT
 81 AACGGCAAGA GTCCCTCCGG ATCCCAGTGC GAGACGTTAG
121 TTACTACGCA AAGGAGGCTC CGGGAGGGTC CGCCACTACC
161 TTTAAGGGCC CACGTCGGCC GTGGGGCCCC AAAACCAAGC
201 GGTGCTTGAG GGTTGAAATG ACGCTCGAAC AGGCATGCTC
241 GCCAGAATGC TGGCGAGCGC AATGTGCGTT CAAAGATTCG
281 ATGATTCACT GAATTCTGCA ATTCACATTA CTTATCGCAT
321 TTCGCTGCGT TCTTCATCGA TGCCAGAACC AAGAGATCCG
361 TTGTTAAAAG TTTTAATTAT TTGCTTGTGC CACTCAGAAG
401 AGACGTCGTG TAAATAGAGT TTGGTTTCCT CCGGCGGGCG
441 CCCCGTCCCC GTGGTGGGGG CCGGCGCCGG GAGGGGAGGC
481 CCGCGAGAGG CTTCCCCTGC CCGCCGAAGC AACGGTTAGG
521 TATGTTCACA AAGGGTTATA GAGCGGTAAC TCAGTAATGA
561 TCCCTCCGCT GGTTCACCAA CGGAGACCTT GTTACGACTT
601 TTACTTCCAG CTGCGTTCAT CCGATGC
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Fig. 2. Complete sequence of part of the ITS II region of *Colletotrichum* spp. isolate Kyungju 3 from infected cv. sweet persimmon.
The CO-1 primer is underlined.

3 consisted of 627 nucleotides. The sequences were compared with those available in the Genbank sequence database, and the ribosomal internal transcribed spacer II of *Colletotrichum* spp. exhibited a 90% homology (data not shown). The sequences were aligned using the Primer[3] output program (Bioneer, Korea) to design proper primers that could specifically amplify *Colletotrichum* spp. One of several primers was then selected and designated CO-1 (5'-ATAA- CCCTTTGTGAACATAC-3').

The pair of primers, CO-1 and ITS 4, were then amplified in the PCR mixture. Healthy leaves and fruits, and leaves and fruits naturally infected with *Colletotrichum* spp. and other pathogens, were used for the PCR detection (Figs. 3A and 3B). As a result, the pair of primers, CO-1 and ITS 4, was used in the PCR mixture to obtain a 500-bp amplified fragment in the DNA from the leaves and fruits infected with *Colletotrichum* spp. However, the primers did not amplify the DNA of any other pathogens isolated from uninfected leaves and fruits (Fig. 3A). Therefore, the CO-1 and ITS 4 primers were shown to specifically identify and detect *Colletotrichum* spp. Next, a probe encoding the isolate Kyungju 3 was hybridized with the PCR products amplified by the CO-1 and ITS 4 primers. Southern hybridization indicated that the *Colletotrichum* spp. isolates exhibited unique dark and thick bands compared to the bands by other pathogens isolated from uninfected leaves and fruits. The two oligonucleotide primers for specific detection were also used to investigate the annealing temperature ranges and detectable genomic DNA concentrations. The annealing temperature was found to be a crucial factor in optimizing product formation. Although the yields were different, they were detectable at a range from 55°C to 61°C on stained agarose gels (data not shown). In addition, this primer set amplified a visible band with 10 pg– 10 μg template DNA (Fig. 4). The identification of most fungi is

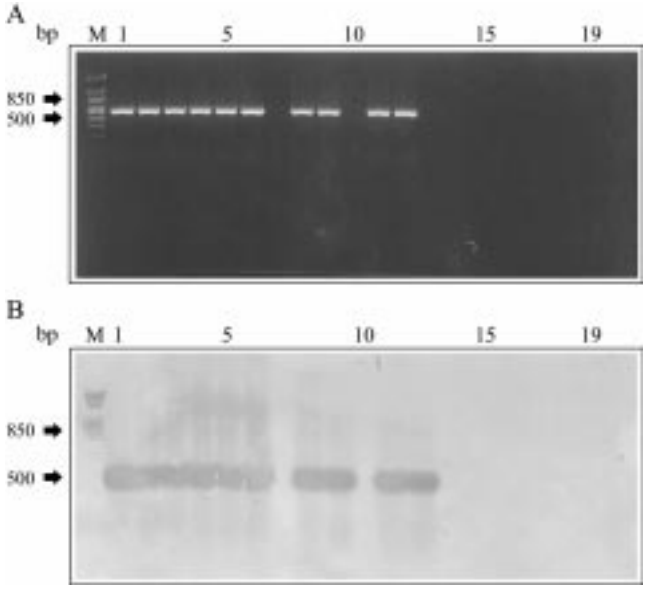

Fig. 3. A. Amplification of 500-bp product using pair of primers CO-1 and ITS 4. B. Result of Southern hybridization with labelled plasmid pGEM^{R}-easy vector containing cloned ITS II region from *Colletotrichum* spp. Lanes: M, 1 kb DNA ladder; 1, *Colletotrichum* spp. isolated in Kyungju; 2, *Colletotrichum* spp. isolated in Kimhae 11; 3, *Colletotrichum* spp. isolated in Milyang 11; 4, *Colletotrichum* spp. isolated in Changwon 4; 5, *Colletotrichum* spp. isolated in Changnyung 16; 6, *Gloeosporium kaki*; 7, Persimmon leaf uninfected with *Colletotrichum* spp.; 8, Persimmon leaf semi-infected with *Colletotrichum* spp.; 9, Persimmon leaf infected with *Colletotrichum* spp.; 10, Persimmon fruit uninfected with *Colletotrichum* spp.; 11, Persimmon fruit semi-infected with *Colletotrichum* spp.; 12, Persimmon fruit infected with *Colletotrichum* spp.; 13, Persimmon fruit uninfected with *Mycospherella*; 14, Persimmon fruit semi-infected with *Mycospherella*; 15, *Pestalophiopsis* sp.; 16, *Rhizoctonia solani*; 17, *Erwinia* sp.; 18, *Phytophthora infestans*; and 19, *Fusarium oxysporum*.

principally based on morphological characteristics. However, for several genera of fungi, including *Colletotrichum*, an accurate species identification can be very difficult and time consuming, because the morphological features vary significantly [6, 21]. The major problems involved in accurately identifying species are due to the facts that most morphological features are common between species, pure culture conditions for identification are difficult to attain, and the observation time is often too long. Therefore, utilization of a PCR is very effective, because it only requires a small amount of DNA and saves time and labor [5, 6, 12, 21, 25].

DNA-based diagnostic methods have already been developed as highly sensitive and species-specific tools. These techniques are very powerful for detecting and identifying the taxonomy of fungi. In particular, PCR-based techniques are much more valuable than convertional methods, because they only require small quantities of DNA and involve little time. They are also usually carried along with negative controls [6, 20, 22, 23, 24]. In the current work, the sequencing and analysis of the ITS regions of *Colletotrichum* spp. allowed the design of a specific PCR primer possible. The primer set, CO-1 (5'-ATAACCCTTTGTGAACATA-3') and ITS 4 (5'-TCCTCCGCTTATTGATTGC-3'), successfully amplified DNA fragments from *Colletotrichum* spp. The specificity of the PCR-based detection method was also verified by the absence of reactivity with DNA from uninfected tissues and other fungal and bacterial pathogens (Figs. 3A and 3B). In addition, the annealing temperature and template DNA quantity was also investigated, since they are factors that can influence the rate and specificity of amplification [19]. Although the theoretical annealing temperature was calculated to be 54°C, temperatures between 55–61°C were found to be suitable for observing the PCR products of *Colletotrichum* spp. on stained agaroses.

These values are significantly higher than the theoritical optimal temperature of 54°C. Furthermore, 10 pg–10 μg of genomic DNA of *Colletotrichum* spp. was found to be sufficient for a detectable PCR amplification (Fig. 4). In conclusion, the application of PCR technology was proven to be effective in detecting *Colletotrichum* spp. both *in vitro* and *in planta*, therefore, it may help in identifying the taxonomy of *Colletotrichum* spp.

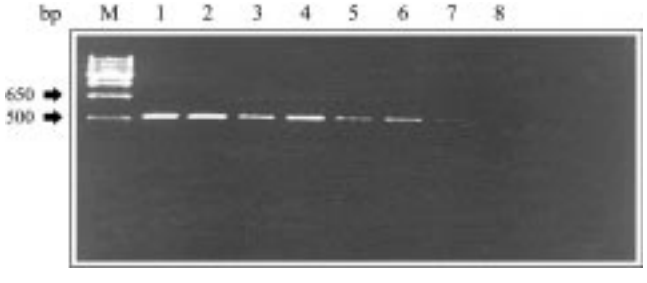

Fig. 4. Amplification products of various amounts of genomic DNA of *Colletotrichum* spp.
Lanes M: molecular marker, amount of genomic DNA, Lanes 1: 10 μg, 2: 1 μg, 3: 100 ng, 4: 10 ng, 5: 1 ng, 6: 100 pg, 7: 10 pg, and 8: 1 pg.

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