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Lee, Seung-Yeol
Laboratory of Forest Resources Management, Division of Agro-environmental Sciences, Department of Forest Environmental Sciences, Faculty of Agriculture, Kyushu University | College of Agriculture and Life Sciences, Kyungpook National University

Lim, Yang-Sook
Laboratory of Forest Resources Management, Division of Agro-environmental Sciences, Department of Forest Environmental Sciences, Faculty of Agriculture, Kyushu University | Persimmon Experiment Station, Gyeongsangbuk-do Agricultural Research & Extension Services

Jung, Hee-Young
Laboratory of Forest Resources Management, Division of Agro-environmental Sciences, Department of Forest Environmental Sciences, Faculty of Agriculture, Kyushu University | College of Agricultural and Life Sciences, Kyungpook National University

Ohga, Shoji
Laboratory of Forest Resources Management, Division of Agro-environmental Sciences, Department of Forest Environmental Sciences, Faculty of Agriculture, Kyushu University

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Nested PCR–based Assay for Diagnosis of Circular Leaf Spot Disease–causing *Mycosphaerella nawae* and its Application

Seung–Yeol LEE[1], Yang–Sook LIM[2], Hee–Young JUNG[1]* and Shoji OHGA

Laboratory of Forest Resources Management, Division of Agro–environmental Sciences,
Department of Forest Environmental Sciences, Faculty of Agriculture,
Kyushu University, Sasaguri, Fukuoka 811–2415, Japan
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In this study, we developed a nested PCR detection method based on draft genomic sequences of *Mycosphaerella nawae* and then applied the method to survey primary symptoms of circular leaf spot (CLS) and the seasonal ascospore scattering period. To develop the assay for detecting the fungus, 11 primers for the initial PCR and 19 primers for the nested PCR were designed (based on the glycoside hydrolase family 3 gene) using draft genomic sequences of *M. nawae*. Consequently, the primer pairs Mn–F1/Mn–R1 and Mn–nes–168f/Mn–nes–483r were selected and their specificities examined using regional *M. nawae* isolates, various healthy persimmon leaves, other fungal pathogens of persimmon, and allied species of *M. nawae*. Furthermore, the developed nested PCR method was applied for the diagnosis of the initial stage of CLS using both diseased and asymptomatic leaves. Additionally, scattered *M. nawae* ascospores collected by a spore sampler were surveyed from May to November 2015. Therefore, it is assumed that the nested PCR method can suggest the proper fungicide spraying period for CLS through anticipation of the ascospore quantity, and the early diagnosis of CLS will enable control of its development on persimmon.

Key words: Circular leaf spot, Diagnosis, *Mycosphaerella nawae*, Nested PCR

INTRODUCTION

Circular leaf spot (CLS) disease, caused by *Mycosphaerella nawae* Hiura et Ikata, has been reported in Japan, Korea, and Spain (Ikata and Hitomi, 1929; Kang *et al*., 1993; Berbegal *et al*., 2010). It is the most destructive disease in persimmon cultivation, causing severe necrotic spots, chlorosis, and red discoloration on leaves, and early defoliation (Vicent *et al*., 2011). Moreover, it leads to premature fruit maturation and abscission, ultimately resulting in economic losses (Kwon and Park, 2004; Berbegal *et al*., 2010). Thus, CLS should be controlled to prevent crop yield and quality losses.

In Korea, the typical symptoms of CLS are observable at around mid–September (Kwon and Park, 2004). Before this period, the initial stage of diseased leaves can be observed; however, it can be hard to diagnose it as being that of CLS disease. Furthermore, *M. nawae* has been shown to have a longer incubation period (approximately 90–120 days) in persimmon compared with that of other pathogens (Kwon and Park, 2004), allowing it to asymptomatically infect persimmon leaves from June to August. For this reason, it is important to develop a detection method for *M. nawae* that can diagnose CLS in both diseased and asymptomatic leaves.

Until recently, the ascospore scattering period of *M. nawae* was typically confirmed by observation of captured ascospores on slide glass traps and of the maturation period of the *M. nawae* pseudothecium (Kwon *et al*., 1995; Kwon *et al*., 1997). However, aside from the difficulty in differentiating *M. nawae* ascospores from other fungal spores, counting the ascospores can be a time–consuming and laborious process. For this reason, the *M. nawae*–specific detection method should not only be able to diagnose CLS but also help in the surveillance of the ascospore scattering periods as well.

The aims of this study were to search for molecular marker genes based on the draft genome sequences of *M. nawae* and use these to develop a *M. nawae*–specific nested PCR method to diagnose CLS disease and detect the fungus. Using the developed nested PCR method, we successfully confirmed the *M. nawae* ascospore scattering periods and detected the initial stage of CLS in asymptomatic diseased leaves.

MATERIALS AND METHODS

Sample collection

Diseased leaves showing typical symptoms of CLS were collected from persimmon orchards located in Sangju–si (SJ) and Gumi–si in Gyeongbuk province, and Gimhae–si, Miryang–si, and Changwon–si in Gyeongnam province in Korea, as well as in Wakayama prefecture in Japan, during the end of September 2014 through to October 2015. To isolate *M. nawae* from the diseased leaves, the part showing CLS symptoms was cut using a blade and then sterilized in 70% ethanol and 1% sodium hypochlorite, and washed 3 times in double–distilled water (DDW). The sterilized samples were dried on a filter paper in room temperature for 30 min. Then, 50 μL of DDW was added to the underside of the area showing the symptoms, and the sample was spread on a potato

[1] College of Agricultural and Life Sciences, Kyungpook National University, Daegu 41566, Korea
[2] Persimmon Experiment Station, Gyeongsangbuk–do Agricultural Research & Extension Services, Sangju 37268, Korea
* Corresponding author (E–mail: ohga@forest.kyushu–u.ac.jp, heeyoung@knu.ac.kr)

dextrose agar (PDA) plate and stored in a 25°C incubator until colonies appeared. After 2–3 days, small dark black colonies were transferred to a new PDA plate. To identify *M. nawae* isolates, PCR amplification was performed in 1× *Taq* reaction buffer containing 0.4 μL of 10 mM dNTP, 1 μL of 10 pM of each ITS 1F/ITS 4 primer pair (White *et al.*, 1990), and 0.2 μL of *Taq* DNA polymerase (Solgent, Daejeon, Korea). The PCR was carried out in a Veriti 96–well thermal cycler (Applied Biosystems, Carlsbad, CA, USA) following conditions previously described (White *et al.*, 1990). Thereafter, the PCR products were purified and directly sequenced by Solgent Co. Ltd. (Daejeon, Korea).

Genome sequencing and primer design for *M. nawae* detection

To search specific regions of the fungus, whole–genome sequencing was conducted using the *M. nawae* SJ isolate. Total genomic DNA extraction from *M. nawae* was conducted by the lithium acetate buffer extraction method (Lõoke *et al.*, 2011). Using the total DNA obtained, genome sequencing was conducted using the PacBio RS Ⅱ sequencer (Seeders, Daejeon, Korea). The specific draft genome sequences obtained were randomly compared with sequences in the NCBI database using the basic local alignment search tool (http://www.ncbi.nlm.nih.gov). Based on the selected specific region, 11 primer sets were designed using the CLUSTAL W software (http://clustalw.ddbj.nig.ac.jp/top–e.html) and compared with other allied species of genus *Mycosphaerella* retrieved from the NCBI database.

Primer specificity and sensitivity

To examine the specificity of the designed primer pair, regional *M. nawae* isolates were examined along with other fungal pathogens of persimmon and allied species of *M. nawae* provided by the Korean Agricultural Culture Collection (Wanju, Korea) and CBS–KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands), as well as various healthy persimmon cultivars collected from Sangju, Korea in 2014 (Table 1). The total genomic DNAs were extracted using the cetyl trimethylammonium bromide (CTAB) method (Namba *et al.*, 1993) and used as template DNA. The PCR amplification was performed in 1× *Taq* reaction buffer containing 0.4 μL of 10 mM dNTP, 1 μL of 10 pM of each forward and reverse primers, and 0.2 μL of *Taq* DNA polymerase. The PCR was conducted in the Veriti 96–well thermal cycler under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 65°C for 60 s, and 72°C for 90 s, with a final extension step at 72°C for 5 min. The PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and observed under an UV illuminator. After the initial PCR, the amplified PCR products were purified using ExoSAP–IT (GE Healthcare, Amersham, Buckinghamshire, UK) and directly sequenced by Solgent. Based on the obtained sequences, inner primer pairs were designed for the nested PCR based on the specificity of *M. nawae*. The designed nested PCR primer pair was examined

Table 1. List of fungal species used in this study

No.	Species	Isolation host	Culture collection accession number	Geographic origin
1	*Botryis cinerea*	*Diospyros kaki*	KACC 41008	Korea
2	*Pestalotiopsis diospyri*	*Diospyros kaki*	KACC 44266	Korea
3	*Phomopsis diospyri*	*Diospyros kaki*	KACC 41009	Korea
4	*Penicillium expansum*	*Diospyros kaki*	KACC 41007	Korea
5	*Colletotrichum acutatum*	*Diospyros kaki*	KACC 45234	Korea
6	*Pseudocercospora kaki*	*Diospyros kaki*	KACC 43176	Korea
7	*Cladosporium cladosporioides*	*Diospyros kaki*	KACC 41011	Korea
8	*Amycosphaerella africana*	*Eucalyptus globulus*	CBS 110500	Australia
9	*Phaeophleospora gregaria*	*Eucalyptus globulus*	CBS 110501	Australia
10	*Paramycosphaerella marksii*	*Eucalyptus globulus*	CBS 110920	Australia
11	*Mycosphaerella keniensis*	*Eucalyptus globulus*	CBS 111001	Kenya
12	*Paramycosphaerella intermedia*	*Eucalyptus saligna*	CBS 114356	New Zealand
13	*Zasmidium parkii*	*Eucalyptus grandis*	CBS 387.92	Brazil
14	*Mycosphaerella madeirae*	*Eucalyptus globulus*	CBS 112895	Portugal
15	*Mycosphaerella stromatosa*	*Protea* sp.	CBS 101953	South Africa
16	*Lecanosticta acicola*	*Pinus caribaea*	CBS 133789	Mexico
17	*Phaeophleospora scytalidii*	*Eucalyptus urophylla*	CBS 118493	Colombia
18	*Passalora fulva*	*Lycopersicon esculentum*	CBS 119.46	Netherlands
19	*Mycosphaerella graminicola*	*Triticum aestivum*	CBS 100329	–
20	*Mycosphaerella musae*	*Musa cultiva*	CBS 121386	Malawi

using the initial PCR products under the same condition as described above. In the case of the sensitivity test, the detection limits of the developed primer pairs were examined using 10–fold serial dilutions, where the DNA of the SJ isolate (set at approximately 8 ng/μL) was diluted to 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, and 1×10^{-6}. PCR was conducted on these diluted samples in the same way as described above. The PCR products were then electrophoresed on a 1% agarose gel, stained with ethidium bromide, and observed under an UV illuminator.

Collection of leaves with initial stage of abnormal symptoms

To examine the developed primer pairs, leaves showing black ring spots (BRSs), healthy leaves collected near diseased (HeDis) leaves, and leaves with CLS disease were collected. The BRS leaves were collected in early August, whereas the CLS and HeDis leaves were collected in October 2015 in SJ, Korea. Over 30 BRS, HeDis, and CLS leaves were collected and observed under a digital microscope (DIMIS–M, Anyang, Korea). Additionally, total DNA was extracted from these leaves by using the CTAB method (Namba, 1993), and was then subjected to PCR using the developed primer pairs. The PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and observed under an UV illuminator.

Detection of *M. nawae* ascospores on spore traps

To apply the detection method to captured ascospores, the nested PCR assay was used to examine the samples trapped on a Burkard 7–day recording volumetric spore sampler (Burkard Scientific Sales, Ltd., Rickmansworth, England). The spore trap was placed in a persimmon field in SJ from the end of May to early November 2015. Sticky double–sided tape was attached onto the glass slide and it was positioned 50 cm above the ground near the center of the field. The ascospores, which were collected at 3–day intervals, were used to extract the total DNA for examination by the nested PCR assay as described above.

RESULTS AND DISCUSSION

Specificity and sensitivity of the designed primer pair

The design of all 11 primer sets for the initial PCR was based on the partial glycoside hydrolase family 3 protein gene, which showed high specificity to *M. nawae*, and each of the primers were tested on *M. nawae* SJ isolates (data not shown). Consequently, the Mn–F1 (5'–GCG GTG AAC TCA CTA AAG CT–3') / Mn–R1 (5'–CTT ATC GTA TGC AGT GAT CTC C–3') primer pair was selected for its specificity. Using this selected primer pair, PCR was conducted using regional *M. nawae* isolates (Fig. 1A). The genomic DNAs of the regional isolates consistently amplified the approximately 600 bp target fragment of the Mn–F1/Mn–R1 primer pair. The amplified fragments were purified and sequenced. As the results, approximately 570 bp was obtained from each of regional isolates, and they were identical each other (data not shown). The obtained sequence was deposited in NCBI Genbank database under following accession number: XXXXXXXX. To check the specificity of this primer pair, it was tested using other pathogens of persimmon, healthy persimmon varieties, and allied species of *M. nawae*. There was no amplification of this fragment observed in the other pathogens and healthy persimmon varieties (Fig. 2A); however, 4 allied species of *M. nawae* (viz., *Phaeophleospora gregaria, Mycosphaerella pseudoellipsoidea, Mycosphaerella stromatosa*, and *Mycosphaerella madeirae*) amplified the same size of DNA fragment (data not shown). To develop the specificity of the inner primer, the amplified DNA fragments were purified and sequenced and used to redesign *M. nawae*–specific regions for nested PCR primer pairs. Among 19 primers tested, the Mn–nes–168f (5'–ATT AAC GAG TAT GTT CCA GTC CGG –3') / Mn–nes–483r

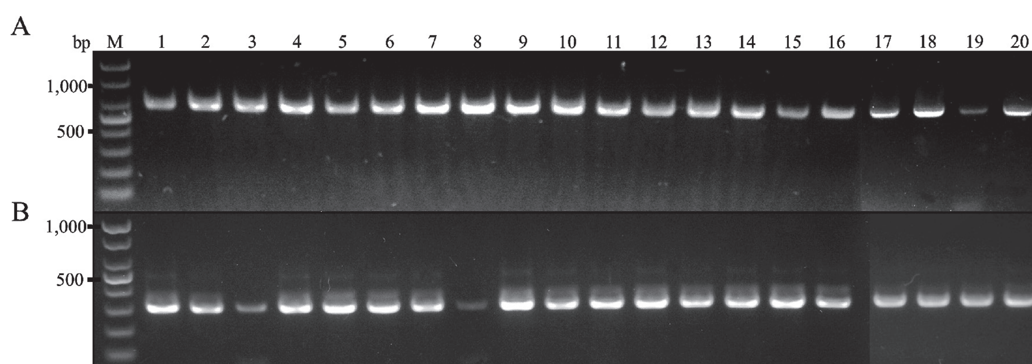

Fig. 1. Results of initial and nested PCRs using total DNA extracted from regional Korean *Mycosphaerella nawae* isolates. A: Results of the initial PCR. B: Results of the nested PCR. Lanes 1–3: isolates CW01, CW02, and CW03 from Changwon–si; 4–6: isolates GM01, GM02, and GM03 from Gumi–si; 7–9: isolates MY01, MY02, and MY03 from Miryang–si; 10–12: isolates KH01, KH02, and KH03 from Gimhae–si; 13–15: isolates Jap01, Jap02, and Jap03 from Japan; and 16–20: isolates SJ01, SJ02, SJ03, SJ04, and SJ05 from Sangju–si. M: 100 bp DNA ladder. The red arrow indicates the targeted fragment.

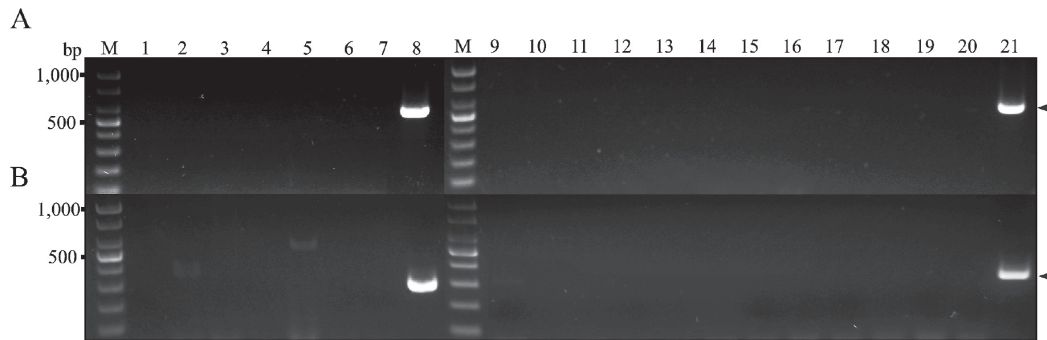

Fig. 2. Results of initial and nested PCRs using total genomic DNA extracted from other fungal pathogens of persimmon and from healthy persimmon cultivars. A: Results of the initial PCR. B: Results of the nested PCR. Lanes 1: *Botrytis cinerea*; 2: *Pestaloptiopsis diospyri*; 3: *Phomopsis diospyri*; 4: *Cladosporium cladosporioides*; 5: *Colletotrichum acutatum*; 6: *Pseudocercospora kaki*; 7: *Penicillium expansum*; 8: total DNA of *Mycosphaerella nawae* isolate SJ03 (positive control); M: 100 bp DNA ladder; 9: Sangju doongsi; 10: Dogeunjosaeng; 11: Chagwang; 12: Gabjubaekmock; 13: Hamansoosi; 14: Soohong; 15: Cheongdo bansi; 16: Dansangsi; 17: Walhasi; 18: Woonpungjunsi; 19: Gyeongsanbansi; 20: Gojongsi; and 21: total DNA of *M. nawae* isolate SJ03 (positive control). The red arrows indicate the targeted fragment.

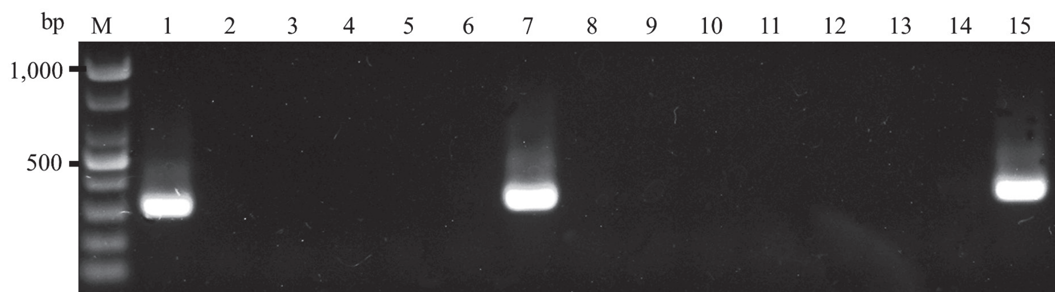

Fig. 3. Results of nested PCRs using total genomic DNA extracted from allied species. Lanes 1: A*mycosphaerella africana*; 2: *Phaeophleospora gregaria*; 3: *Paramycosphaerella marksi*i; 4: *Mycosphaerella ellipsoidae*; 5: *Mycosphaerella keniensis*; 6: *Paramycosphaerella intermedia*; 7: *Zasmidium parkii*; 8: *Mycosphaerella madeirae*; 9: *Mycosphaerella stromatosa*; 10: *Lecanosticta acicola*; 11: *Phaeophleospora scytalidii*; 12: *Passalora fulva*; 13: *Mycosphaerella gregaria*; 14: *Mycosphaerella musae*; and 15: total DNA of *Mycosphaerella nawae* isolate SJ03 (positive control).

(5'– GTG CGA CCA GAT GCG GAA –3') primer pair was selected for its specificity. Using the primer pair, nested PCR amplicons were uniformly produced by the regional *M. nawae* isolates, whereas no amplicons were observed in the other persimmon pathogens and healthy persimmon cultivars (Fig. 1B and 2B). However, among the 14 allied species, *Amycosphaerella africana* and *Zasmidium parkii* amplified (Fig. 3) a fragment of the same size as *M. nawae*. In a previous study that used the primer pair Mnf/Mnr (based on the ITS region) for nested PCR detection of *M. nawae* on persimmon leaves, the authors reported that there were no amplifications of the PCR product using various pathogens and *Mycosphaerella* sp. (Berbegal *et al.*, 2013). However, 14 *M. nawae* allied species used in this study amplified fragments of the same size as that from *M. nawae* (data not shown). In the case of *A. africana* and *Z. parkii*, which amplified the fragment using Mn–nes–168f/Mn–nes–483r, both fungal species have been reported only in limited regions such as Columbia and Australia and have common hosts such as *Eucalyptus* spp. or *Protea* sp.

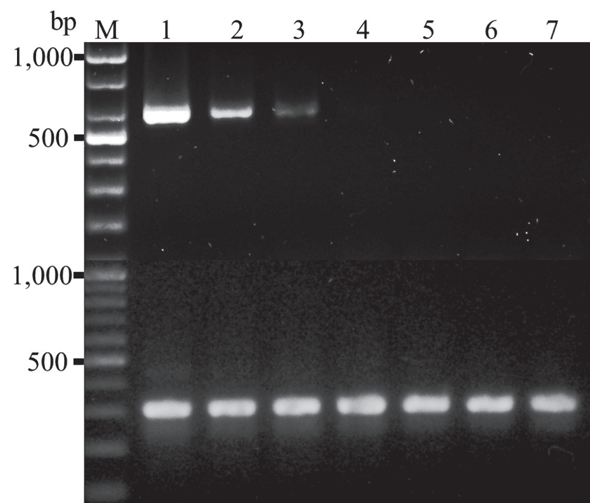

Fig. 4. Results of the sensitivity test using diluted genomic DNA from the *Mycosphaerella nawae* Sangju–si isolate. Lanes 1: 10^0 (8 g/μL); 2: 10^{-1} dilution; 3: 10^{-2} dilution; 4: 10^{-3} dilution; 5: 10^{-4} dilution; 6: 10^{-5} dilution; and 7: 10^{-6} dilution.

(Quaedvlieg *et al*., 2014) and have not been reported in persimmon. Moreover, both fungal species have not been reported in Korea (KSPP, 2009). Because there was no amplification of the PCR product in the other persimmon pathogens and in various healthy cultivars, there would be no problem in using these developed initial and nested PCR primer pairs for the diagnosis of CLS. The sensitivity of Mn–F1/Mn–R1 and of Mn–nes–168f/Mn–nes–483r was evaluated using a 10–fold dilution series of DNA from the *M. nawae* SJ isolate. The initial PCR could amplify fragments with the 10^{-2}–fold dilution of genomic DNA, whereas the nested PCR could amplify fragments with the 10^{-6}–fold dilution (Fig. 4). From these results, it can be assumed that the developed nested PCR method can detect low concentrations of *M. nawae* infection.

Application of developed primer pairs in CLS–diseased leaves

To apply the developed primer pairs, total DNA samples extracted from CLS–diseased, BRS, and HeDis leaves were examined. Before DNA extraction, the BRS samples were examined under a digital microscope, where a brown spot surrounded by a black ring with a yellow halo could be seen (Fig. 5). Although the size of the symptoms differed among the samples, the symptom features were consistently similar (Fig. 5). Initial and nested PCRs were conducted using the CLS, BRS, and HeDis DNA samples and the developed primer pairs. In the case of the initial PCR using Mn–F1/Mn–R1, targeted DNA

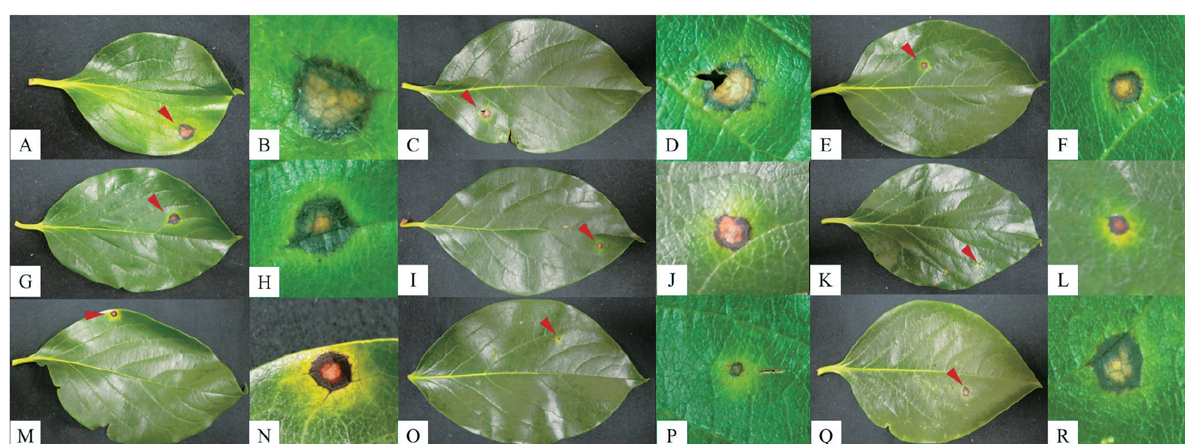

Fig. 5. Various abnormal leaves with black ring spot (BRS) symptoms. Leaves were collected from the Sangju-si (SJ) region in August 2015. Red arrowheads indicate BRS symptoms. A: SJ-32; B: enlarged image of panel A; C: SJ-31; D: enlarged image of panel C; E: SJ-38; F: enlarged image of panel E; G: SJ-33; H: enlarged image of panel G; I: SJ-36; J: enlarged image of panel I; K: SJ-36; L: enlarged image of panel K; M: SJ-34; N: enlarged image of panel M; O: SJ-37; P: enlarged image of panel O; Q: SJ-39; and R: enlarged image of panel Q. Red arrowheads indicate the symptoms.

Table 2. Results of initial and nested PCR assays applied in various types of collected leaves and in monthly surveyed samples collected using a spore sampler in 2015

Type of collected leaves from SJ in 2015	No. of positive result of initial PCR/total samples	No. of positive result of nested PCR/total samples
Brown ring spot (BRS)	0/9	9/9
Healthy leaves collected in infected trees (HeDis)	0/13	13/13
Circular leaf spot (CLS) diseased leaves	5/7	7/7
Monthly surveyed samples collected using spore sampler in 2015	**No. of positive result of initial PCR/total samples**	**No. of positive result of nested PCR/total samples**
May	2/21	14/21
June	0/9	8/9
July	0/14	11/14
August	0/30	11/30
September	0/30	2/30
October	0/30	30/31
November	0/3	2/3

fragments were observed in CLS–diseased leaves (Table 2). In the case of the nested PCR using Mn–nes–168f/Mn–nes–483r, the PCR product was amplified from all three types of leaves (Table 2). It has been reported that the typical symptoms of CLS generally start to occur at early September in Korea (Kwon *et al.*, 2004). However, the targeted fragment was observed in BRS leaves that were collected in early August; hence, it can be assumed that those abnormal BRSs are an initial stage of CLS on persimmon. In the case of HeDis, although symptoms were not observed on these leaves, the developed primer pair could detect *M. nawae* in the asymptomatic leaves. Several pathogens have been reported to cause foliar diseases on persimmon in Korea (KSPP, 2009). Among those diseases, the symptoms of angular leaf spot caused by *Pseudocercospora kaki* and leaf blight caused by *Pestalotiopsis diospyri* are similar to those of the initial stage of CLS, and their occurrence periods are overlapped (Chang *et al.*, 1996). For this reason, the accurate diagnose of CLS among various diseases is essential for fungicide spraying or disease incidence surveillance. Therefore, a nested PCR method that can accurately diagnose CLS is essential to reduce the economic effect caused by this disease.

Detection of *Mycosphaerella nawae* ascospores on spore traps

The total DNA samples extracted from ascospores trapped on the sticky double–sided tape were examined using the developed PCR methods. The initial and nested PCR amplification results for seasonal *M. nawae* ascospores collected from spore samplers are shown in Table 2. Ascospores were detected by nested PCR from early May, and were continuously detected until November 2015. Except for two samples detected using initial PCR in May, all other samples were detected using nested PCR. A high detection rate was maintained from May to July, but it decreased in August and September and increased again in October and November (Table 2). According to Kwon and Park (Kwon and Park, 2004), ascospores are released from mid–May to early July in Korea. The nested PCR results showed a similar trend, where detection began in May, increased from June to August, and decreased in September (Table 2). The ascospore observation and PCR detection results were well correlated. The ascospores detected from May to August are thought to originate from pseudothecia in overwintered diseased leaves, whereas those detected from October represent a secondary inoculum originating from the CLS spots (Kwon and Park, 2004).

Generally, predictions of the primary infection period and disease incidence have relied on conventional methods such as counting the number of ascospores or conidia captured by spore traps and observing the appearance of typical symptoms on the host. Counting the number of ascospores or conidia captured on spore traps has been widely used for surveying disease etiology and the dispersal pattern of ascospores and conidia (Falacy *et al.*, 2007; Klosterman *et al.*, 2014; Jones *et al.*, 2015). However, microscopic observation of the tapes from the surveyed spore traps can be time consuming and laborious, and has limited ability to identify the inoculum (Jones *et al.*, 2015). Thus, molecular biological assays based on PCR techniques have been developed to survey the inoculum, including ascospores and conidia (Falacy *et al.*, 2007; Klosterman *et al.*, 2014; Jones *et al.*, 2015). In Korea, *M. nawae* ascospore dispersal patterns have been surveyed to predict the incidence of CLS in agricultural institutions. Although counting the number of ascospores or conidia can confirm their existence, this method is limited and can be insufficient for confirming the presence of *M. nawae*. Thus, the presence of an inoculum can be accurately identified in persimmon orchards using our developed nested PCR assay.

In conclusion, a nested PCR assay was developed that can be used to detect *M. nawae* on persimmon leaves and to diagnose the initial stage of CLS disease. It has proved to be a useful assay for the detection of *M. nawae*. Further studies will be carried out to survey regional primary infection periods and CLS incidence using the developed nested PCR assay.

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