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Research Article

Development of SCAR Markers for the Identification of *Phytophthora katsurae* Causing Chestnut Ink Disease in Korea

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Abstract Sequence characterized amplified region (SCAR) markers are one of the most effective and accurate tools for microbial identification. In this study, we applied SCAR markers for the rapid and accurate detection of *Phytophthora katsurae*, the casual agent of chestnut ink disease in Korea. In this study, we developed seven SCAR markers specific to *P. katsurae* using random amplified polymorphic DNA (RAPD), and assessed the potential of the SCAR markers to serve as tools for identifying *P. katsurae*. Seven primer pairs (SOPC 1F/SOPC 1R, SOPC 1-1F/SOPC 1-1R, SOPC 3F/SOPC 3R, SOPC 4F/SOPC 4R, SOPC 4F/SOPC 4-1R, SOPD 9F/SOPD 9R, and SOPD 10F/SOPD 10R) from a sequence derived from RAPD fragments were designed for the analysis of the SCAR markers. To evaluate the specificity and sensitivity of the SCAR markers, the genomic DNA of *P. katsurae* was serially diluted 10-fold to final concentrations from 1 mg/mL to 1 pg/mL. The limit of detection using the SCAR markers ranged from 100 μg/mL to 100 ng/mL. To identify the limit for detecting *P. katsurae* zoospores, each suspension of zoospores was serially diluted 10-fold to final concentrations from 10×10^5 to 10×10^1 zoospores/mL, and then extracted. The limit of detection by SCAR markers was approximately 10×10^1 zoospores/mL. PCR detection with SCAR markers was specific for *P. katsurae*, and did not produce any *P. katsurae*-specific PCR amplicons from 16 other *Phytophthora* species used as controls. This study shows that SCAR markers are a useful tool for the rapid and effective detection of *P. katsurae*.

Keywords Chestnut ink disease, *Phytophthora katsurae*, Random amplified polymorphic DNA, Sequence characterized amplified region

Since 2005, damage to chestnut cultivation by chestnut ink disease has been reported in the southern chestnut cultivation area of Korea [1]. The pathogen behind this disease has been identified as the soil-borne *Phytophthora katsurae*. *P. katsurae* has multiple hosts and can infect them in multiple regions [2]. Consequently, this pathogen may serve as a limiting factor for chestnut cultivation [3]. The initial symptoms of chestnut ink disease are the extrusion of black liquid and necrosis in the stem. The damaged tree eventually dies, displaying crown dieback and releasing immature fruit [2]. To date, four species of the genus *Phytophthora* have been reported to cause chestnut ink disease: *P. cambivora*, *P. cinnamomi*, *P. cryptogea*, and *P. katsurae*. Of these four species, all except for *P. katsurae* have been reported to cause severe damage to chestnut plantations throughout Europe, the United States, and southern Australia. The American chestnut [*Castanea dentata* (Marsh.)], most of which is planted in most regions of Europe and the United States, is susceptible to these pathogenic fungi [2]. In contrast, the Japanese chestnut (*Castanea crenata*), most of which is widely planted and cultivated in Korea, is susceptible to *P. katsurae*, and this fungal pathogen has severely damaged chestnut cultivation. To prevent the spread of this pathogen to healthy hosts and disease-free regions, a rapid, simple and accurate or reliable identification system of *P. katsurae* will be required for effective disease management because of its wide host range, broad distribution and economic impact. Identification by morphological features has limitations, including laborious

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work, a high time consumption, requirement for microscopic techniques and a comprehensive knowledge of fungal pathogens. Molecular-based tools, such as a DNA-based system, can quickly and easily achieve an adequate database of reference strains [4, 5]. Due to genotype-specific variation, the internal transcribed spacer (ITS) region has been widely used in the design of species-specific PCR primers, and has proven useful in the development of diagnostic tests for the detection of these pathogenic species [5, 6]. However, in the case of the genus *Phytophthora*, some studies have found that using the ITS region for diagnostic purposes is not always appropriate, as in some cases it is still difficult to differentiate among closely related species using the ITS sequences [5, 7, 8]. This difficulty in designing specific primers is the result of insufficient variation in the ITS sequence of *Phytophthora* species [9, 10]. Although some authors have reported other multicopy genes, including the mitochondrial cytochrome oxidase (*cox*) gene and the *Ypt1* gene as alternatives to ITS regions, in some cases, it is difficult to distinguish hybrid species and closely related *Phytophthora* species e.g., *P. cactorum/P. idaei*, and *P. iliciss/P. nemerosa* [11, 12]. An alternative approach would be to develop specific primers using random amplified polymorphic DNA (RAPD) since RAPD can be applied for the design of species or strain-specific markers from randomly amplified sequences [13, 14]. Thus, sequence characterized amplified region (SCAR) markers specific to fungal pathogens can be derived from the sequence of a specific strain or gene, or from sequences randomly amplified by decamers [15]. In this study, we aimed to develop specific SCAR markers for *P. katsurae* and to apply them for the identification of this fungal pathogen.

MATERIALS AND METHODS

Fungal isolates and inoculation. All fungal isolates used in this study, ten isolates of *P. katsurae* and 16 isolates of other *Phytophthora* species, including destructive plant or tree pathogens causing severe damage in chestnut trees (as well as a wide range of economically important cultivated plants), were obtained from the Tree Pathology and Mycology Lab (Kangwon National University, Chuncheon, Korea) and the Korean Agricultural Culture Collection (Suwon, Korea). However, in the case of *P. katsurae*, only isolates from Korea were included for this study, as low variance between these isolates of *P. katsurae* has been proven by Oh [1]. The obtained fungal strains were cultured on V8 medium [16], a *Phytophthora*-selective medium covered with a sterilized cellulose membrane (Cellu-Sep T4; Membrane Filtration Products, Inc., Segiun, TX, USA) to facilitate the collection of mycelium and stored at 25°C in the dark [3]. The list of fungal isolates is shown in Table 1.

Genomic DNA extraction. To extract genomic DNA from *P. katsurae*, 1 mg of mycelium was scraped from a cellulose membrane after 1 wk of incubation at 25°C, and freeze-dried in a chamber at −20°C for further study. Genomic DNA was extracted using a DNeasy Plant mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Genomic DNA from *P. katsurae* zoospores was extracted using a Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions, with slight modifications. To gather zoospores of *P. katsurae* at the bottom of a 1.5 mL microtube, 1 mL of zoospore suspension was vigorously vortexed for 1 min. Each sample was then immediately transferred to a 2.0 mL Power Soil bead tube.

Table 1. List of *Phytophthora* species used in this study

Strain No.	Species	Source	Origin
TPML 06522	*Phytophthora katsurae*	TPML	Korea
TPML 08001	*P. katsurae*	TPML	Korea
TPML 08002	*P. katsurae*	TPML	Korea
TPML 08003	*P. katsurae*	TPML	Korea
TPML 08004	*P. katsurae*	TPML	Korea
TPML 08005	*P. katsurae*	TPML	Korea
TPML 08007	*P. katsurae*	TPML	Korea
TPML 08008	*P. katsurae*	TPML	Korea
TPML 08009	*P. katsurae*	TPML	Korea
TPML 08010	*P. katsurae*	TPML	Korea
TPML 07001	*P. citricola*	TPML	Korea
TPML 07004	*P. cactorum*	TPML	Korea
TPML 07005	*P. cinnamomi*	TPML	Korea
TPML 07006	*P. cambivora*	TPML	Korea
TPML 07007	*P. lateralis*	TPML	Korea
KACC 40173	*P. boehmeriae*	KACC	Korea
KACC 40185	*P. citrophthora*	KACC	Korea
KACC 40161	*P. cryptogea*	KACC	Korea
KACC 40190	*P. drechsleri*	KACC	Korea
KACC 40711	*P. gonapodyides*	KACC	Korea
KACC 40712	*P. erythroseptica*	KACC	Korea
KACC 40718	*P. infestans*	KACC	Korea
KACC 40194	*P. melonis*	KACC	Korea
KACC 40402	*P. nicotianae*	KACC	Korea
KACC 40409	*P. palmivora*	KACC	Korea
KACC 40468	*P. sojae*	KACC	Korea

TPML, Tree Pathology and Mycology Lab Knagwon National University; KACC, Korea Agricultural Culture Collection.

Development and detection of *P. katsurae* zoospores. To produce zoosporangium, agar discs with actively growing mycelium at the margin of the culture colony were cut off using an 8 mm cork borer and transferred into a 90-mm-diameter Petri dish containing soil extraction solution [17]. *P. katsurae* zoospores were released according to Lee *et al.* [3] with slight modifications. The culture was incubated in a growth chamber at 25°C for 10~14 days with light (1,000 lux). The culture was chilled at −4°C for 1 hr in the dark after transplanting the small pieces into a 35-mm-diameter Petri dish containing soil extraction solution. This dish was then incubated at 25°C for 2 hr. Zoospore release was achieved by incubating the dish at 25°C for 2 hr in the dark. The zoospore concentration was determined using a

hemacytometer, and the zoospore suspension was serially diluted 10-fold to final concentrations ranging from 10×10^5 to 10×10^1 zoospores/mL.

Amplification conditions for RAPD analysis. RAPD amplification was performed in a total reaction volume of 50 μL containing 2 μL of genomic DNA, 1 μL (10 pM) of random primer (Operon, Inc., Huntsville, AL, USA), 2.5 units of Taq polymerase (Takara Shuzou Co. Ltd., Otsu, Japan), 4 μL (2.5 mM) of the dNTP mixture, and 5 μL of 10× Ex Taq buffer (+20 mM Mg^{2+}). The amplification reaction was conducted on a My Cycler thermal cycler system (BioRad, Hercules, CA, USA). The cycling profile for RAPD analysis consisted of an initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 37°C for 30 sec, and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. This experiment was conducted at least three times to ensure the reproducibility of results. The amplification products were analyzed by electrophoresis (Mupid-21. Cosmo Bio Ltd., Tokyo, Japan) on a 1.5% (w/v) agarose gel in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.0), and then visualized using a UV transilluminator (Gel Documentation System; Bio-Rad) after staining with ethidium bromide (10 μg/mL) for 30 min.

RAPD analysis. RAPD analysis of the total DNA extracted from *P. katsurae* was conducted using 40 random decamer primers (Operon, Inc., Huntsville, AL, USA). PCR amplification was performed with the OPC and OPD primer sets (Operon, Inc., Huntsville, AL, USA) as described above. RAPD analysis of two *P. katsurae* isolates, TPML06522 and TPML08001, and 16 other *Phytophthora* species was conducted. The RAPD profiles were specific to each *Phytophthora* species, and seven DNA fragments distinguishing *P. katsurae* from the other 16 *Phytophthora* species were screened. The *P. katsurae*-specific DNA fragments were directly eluted from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and ligated into a pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. The plasmids were used to transform *Escherichia coli* strain JM 109 competent cells (Promega, Madison, WI, USA) and the recombinant colonies were identified by blue-white color selection after 15 hr of growth at 37°C on Luria broth (LB) agar medium (1.8% trypton-NaCl, 0.5% yeast extract, 1.6% agar) containing ampicillin, 20% IPTG (isopropyl b-D-1-thiogalactopyranoside), and 2% X-Gal (5-bromo-4-chloro-3-indolyl b-D-galactopyranoside) following the manufacturer's recommendations. Plasmids were purified from LB/ampicillin liquid cultures of the selected colonies using a Wizard Miniprep DNA Purification System (Promega, Madison, WI, USA). Purified plasmid DNA was sequenced using an ABI PRISM 3730XL Analyzer Sequencer (Applied Biosystems, Foster City, CA, USA). All sequences obtained from this study were deposited in GenBank with the following the accession numbers, JQ796817 to JQ796822.

Table 2. *Phytophthora katsurae*-specific markers designed for this study

Primer	Sequences	Size (bp)
SOPC 1F	42 65 nt 5′ GTG AGG CGT CGA AGA ACG AGG AAT 3'	1,209
SOPC 1R	1,228 1,251 nt 5′ GTG AGG CGT CCG GTG TAG AAG AGG 3'	
SOPC 1-1F	42 65 nt 5′ GTG AGG CGT CGA CGA GGC AGA ACA 3'	1,100
SOPC 1-1R	1,120 1,142 nt 5′ GTG AGG CGT CCA ACA GGA TGC TT 3'	
SOPC 3F	42 59 nt 5′ GAT GAC CGC CCC AGA AGG 3'	1,120
SOPC 3R	1,143 1,162 nt 5′ GAT GAC CGC CGT GTT GAT GG 3'	
SOPC 4F	5′ AAA GCT GCG GAC ACG GAG GGG GTA 3'	
SOPC 4R	5′ GCT GCG GCA TAT CTG TGC AAG AAC TCG3'	
SOPC 4-1R	5′ AAA GCT GCG GCA TAT CTG TGC A 3'	
SOPD 9F	46 68 nt 5′ GAG AGC CAA CAT GAG AGT GAA GA 3'	1,424
SOPD 9R	1,449 1,470 nt 5′ TGA GAG CCA ACG CGG AGG TAA G 3'	
SOPD 10F	42 62 nt 5′ ACC CGG TCA CGG ATG AGG AGG 3'	1,718
SOPD 10R	1,739 1,760 nt 5′ ATT ACC CGG TCA CTC GGC AGC C 3'	

The underlined sequence represents the original random decamer primer sequence used in the random amplified polymorphic DNA reaction.

Table 3. PCR protocol and condition used in this study

Primer set	Initial denaturation	Denaturation	Annealing	Extension	Cycle	Final extension
SOPC 1F/1R	95°C, 2 min	95°C, 1 min	60°C, 1 min	95°C, 1 min	30	72°C, 5 min
SOPC 1-1F/1-1R	95°C, 2 min	95°C, 1 min	60°C, 1 min	95°C, 1 min	30	72°C, 5 min
SOPC 3F/3R	95°C, 2 min	95°C, 1 min	53°C, 1 min	95°C, 1 min	35	72°C, 5 min
SOPC 4F/4R	95°C, 2 min	95°C, 1 min	60°C, 1 min	95°C, 1 min	35	72°C, 5 min
SOPC 4F/4-1R	95°C, 2 min	95°C, 1 min	60°C, 1 min	95°C, 1 min	35	72°C, 5 min
SOPD 9F/9R	95°C, 2 min	95°C, 1 min	55°C, 1 min	95°C, 1 min	35	72°C, 5 min
SOPD 10F/10R	95°C, 2 min	95°C, 1 min	65°C, 1 min	95°C, 1 min	25	72°C, 5 min

Design of SCAR markers and the SCAR reaction. SCAR markers derived from RAPD markers specific to *P. katsurae* were designed by conversion of the selected RAPD markers into SCAR markers. Seven sets of the SCAR markers designed and used in this study are listed in Table 2. The SCAR reaction was carried out in a total volume of 25 μL containing 1 μL of genomic DNA, 1 μL of upstream primer (20 pM), 1 μL of downstream primer (20 pM), 5 μL of 10× Taq buffer, 0.5 μL of 10 mM dNTPs, and 2.5 units of Taq polymerase (Promega, Madison, WI, USA). A PCR assay with the SCAR markers was performed according to the method described in Table 3.

Primer specificity and sensitivity. To evaluate the specificity of the designed SCAR markers, seven sets of the SCAR markers, SOPC 1F/SOPC1R, SOPC 1-1F/SOPC 1-1R, SOPC 3F/SOPC 3R, SOPC 4F/SOPC 4R, SOPC 4F/SOPC 4-1R, SOPD 9F/SOPD 9R, and SOPD 10F/SOPD 10R designed in this study were tested against two isolates of *P. katsurae* and 16 isolates of other *Phytophthora* species as the controls. To assess the sensitivity of the specific markers, all genomic DNA extracted from a pure mycelium of *P. katsurae* was serially diluted 10-fold to final concentrations ranging from 1 mg/mL to 1 pg/mL.

RESULTS AND DISCUSSION

RAPD analysis. RAPD is an easy and rapid method widely used [18, 19] for the analysis of genetic diversity among species of *Phytophthora*, as well as for discriminating genotypes among different species of *Phythophthora*, because this method does not require any information on the sequence data of fungal pathogens. Moreover, Hadrys *et al.* [20] demonstrated that this method might be useful for the identification of fungal isolates and applications to molecular ecology. However, in the case of *Phytophthora* species, the design of species-specific primers based on the rDNA ITS region may be unprofitable because of inter-species variation in this part of the sequence [21, 22]. However, RAPD facilitates the confirmation of variation because it can be applied to the entire genome when comparing genetic variation among fungal pathogens at the species or genera level. Thus, RAPD should be a reliable alternative for constructing species-specific primers, including SCAR makers. This method was thus applied in our study for developing specific SCAR markers. Forty random decamer primers (Operon, Inc., Huntsville, AL, USA) were evaluated using *P. katsurae* isolates in PCR reactions. Among the 40 random decamer primers tested in this study, five (OPC 02, OPC 05, OPC 11, OPD 18, and OPD 20) produced band patterns specific to *P. katsurae* (data not shown). PCR amplification with these five random decamer primers was carried out using DNA from two isolates of *P. katsurae* and 16 isolates of other *Phytophthora* species. Each random decamer primer produced approximately 20~25 amplicons (data not shown). The RAPD bands shown to be unique to *P. katsurae*, with sizes of 1,209 bp (OPC 02), 1,120 bp (OPC 05), 2,100 bp (OPC 11), 1,424 bp (OPD 18), and 1,718 bp (OPD20), were selected and used to design SCAR markers. All SCAR markers designed in this study are listed in Table 2.

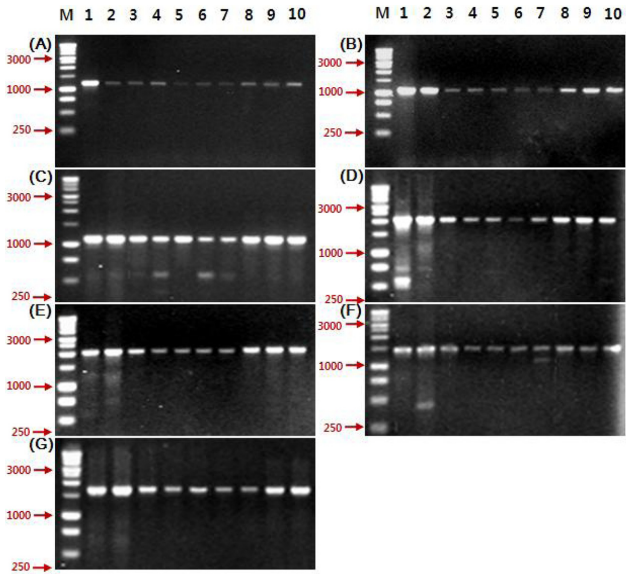

Fig. 1. Sequence characterized amplified regions PCR analysis of *Phytophthora katsurae* amplified using the primers SOPC 1F/1R (A), SOPC 1-1F/1-1R (B), SOPC 3F/3R (C), SOPC 4F/4R (D), SOPC 4F/4-1R (E), SOPD 9F/ 9R (F), and SOPD 10F/10R (G), respectively. "M" represents the 1 kb ladder. Lane 1, TPML 06522; lane 2, TPML 08001; lane 3, TPML 08002; lane 4, TPML 08003; lane 5, TPML 08004; lane 6, TPML 08005; lane 7, TPML 08007; lane 8, TPML 08008; lane 9, TPML 08009; lane 10, TPML 08010.

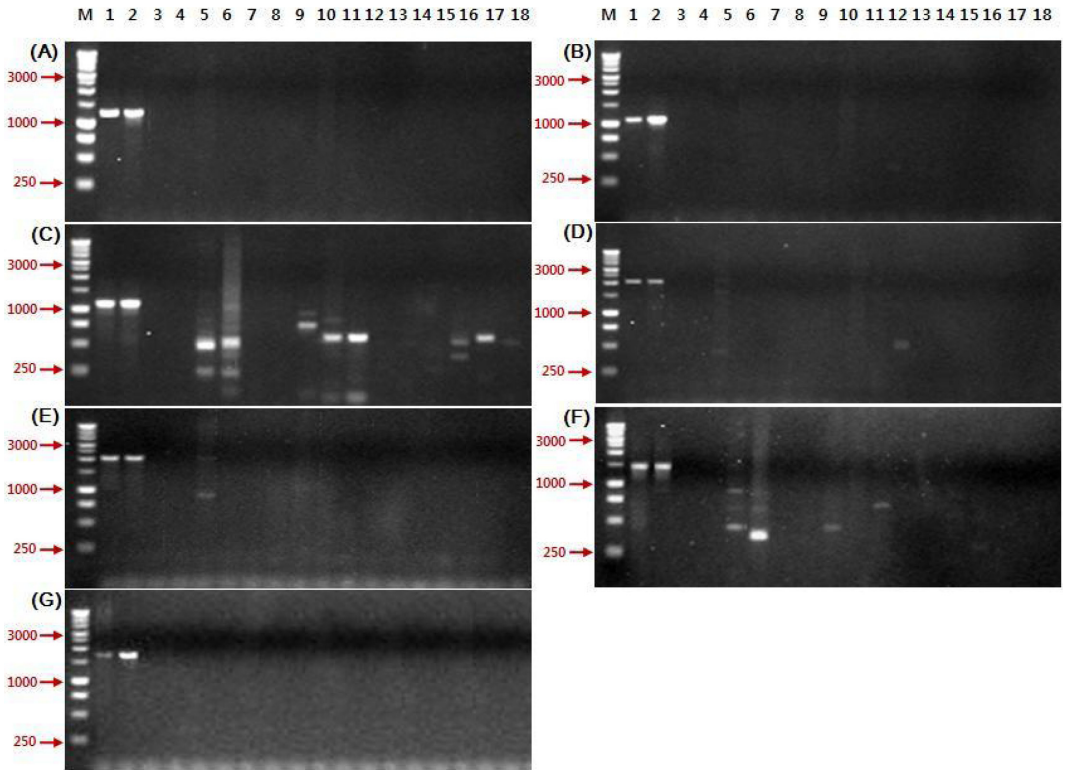

Fig. 2. Sequence characterized amplified regions PCR analysis of *Phytophthora* amplified using the primers SOPC 1F/1R (A), SOPC 1-1F/1-1R (B), SOPC 3F/3R (C), SOPC 4F/4R (D), SOPC 4F/4-1R (E), SOPD 9F/9R (F), and SOPD 10F/10R (G), respectively. "M" represents the 1 kbp ladder. Lane 1, TPML 06522; lane 2, TPML 08001; lane 3, TPML 07001; lane 4, TPML 07004; lane 5, TPML 07005; lane 6, TPML 07006; lane 7, TPML 07007; lane 8, KACC 40173; lane 9, KACC 40185; lane 10, KACC 40161; lane 11, KACC 40190; lane 12, KACC 40402; lane 13, KACC 40711; lane 14, KACC 40718; lane 15, KACC 40194; lane 16, KACC 40712; lane 17, KACC 40468; lane 18, KACC 40409.

Specificity of the SCAR markers for *P. katsurae*. PCR amplification of ten isolates of *P. katsurae* (Fig. 1) and 16 isolates of other *Phytophthora* species as controls (Fig. 2) was performed with SCAR markers. All SCAR markers produced amplicons specific to *P. katsurae* with sizes of 1,209 bp (SOPC 1F/1R), 1,100 bp (SOPC 1-1F/1-1R), 1,120 bp (SOPC 3F/3R), 2,100 bp (SOPC 4F/4R), 2,100 bp (SOPC 4F/4-1R), 1,424 bp (SOPD 9F/9R), and 1,718 bp (SOPD 10F/10R). Furthermore, when PCR products amplified by SCAR markers were sequenced and compared with the sequence used to design SCAR markers, the DNA sequences were identical to each other. In contrast, PCR amplification with SCAR markers did not produce amplicons for the 16 isolates of other *Phytophthora* species. In addition, bands non-specific to *P. katsurae* (Fig. 2C~F) did not correspond to any *P. katsurae* sequence. These results suggest that the detection of *P. katsurae* using SCAR markers is rapid and accurate. The detection and identification of fungal pathogens based on molecular methods using primers derived from the ITS region of rDNA have been widely used in recent years [5, 23]. However, in some cases, species-specific primers based on the ITS sequences are unable to differentiate closely related species. Gomez-Alpizar *et al.* [24] and Kroon *et al.* [25] reported that *P. infestans*, *P. phaseoli*, *P. ipomoeae*, *P.* sp. '*andina*', and *P. mirabilis* are closely related species and have a 99.9% homology in ITS sequence. The same situation has been shown between *P. katsurae* and *P. heveae*, which belong to the same clade [4], and have up to 99.9% similarity in ITS sequence. In addition, Schena and Cooke [26] have also shown that *P. katsurae* is closely related with *P. quercina and P. palmivora* when analyzing phylogenetic relationships using DNA sequence data from four spacers between mitochondrial genes. Due to these limitations in identification using the ITS sequences, SCAR markers might be an alternative method for overcoming these restrictions. In addition, the isolation and identification of fungal pathogens using morphology-based methods is inefficient due to the long growth periods and the requirement of comprehensive knowledge about fungi. To overcome these limitations, alternative methods for constructing PCR primers have been developed and applied to detect *Phytophthora* species in recent years [13, 15], and some authors have reported that SCAR markers could be used as reliable and efficient tools for the differentiation of closely related species [15, 27].

Sensitivity of SCAR markers. All SCAR markers designed for this study were evaluated using genomic DNA extracted from *P. katsurae* mycelia ranging in concentration from

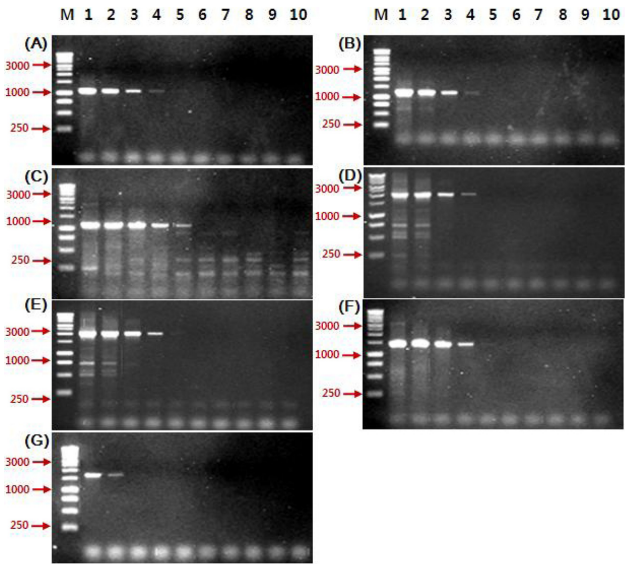

Fig. 3. Sensitivity of sequence characterized amplified regions PCR on *Phytophthora katsurae* mycelium using the primers SOPC 1F/1R (A), SOPC 1-1F/1-1R (B), SOPC 3F/3R (C), SOPC 4F/4R (D), SOPC 4F/4-1R (E), SOPD 9F/9R (F), and SOPD 10F/10R (G), respectively. "M" represents the 1 kb ladder. Lane 1~10, amplified product from DNA at the concentration of 1 mg/mL, 100 μg/mL, 10 μg/mL, 1 μg/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, 100 pg/mL, 10 pg/mL, and 1 pg/mL, respectively.

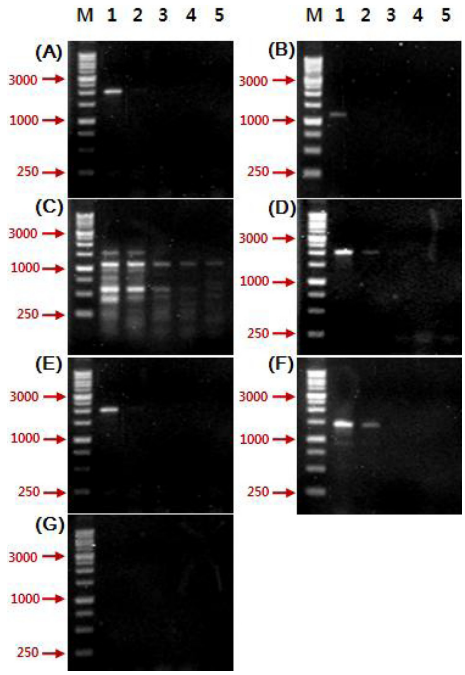

Fig. 4. Sensitivity of sequence characterized amplified regions PCR on zoospore suspensions of *Phytophthora katsurae* using the primers SOPC 1F/1R (A), SOPC 1-1F/1-1R (B), SOPC 3F/3R (C), SOPC 4F/4R (D), SOPC 4F/4-1R (E), SOPD 9F/9R (F), and SOPD 10F/10R (G), respectively. "M" represents the 1 kb ladder. Lane 1~5, amplified product from DNA at concentrations of 10×10^5, 10×10^4, 10×10^3, 10×10^2, and 10×10^1 zoospores/mL, respectively.

1 mg/mL to 1 pg/mL. The limit of detection of the SCAR markers was approximately 1 μg/mL, with two exceptions (Fig. 3). One exception was the PCR assay using the SOPC 4F/4-1R marker, which had a sensitivity of 100 ng/mL (Fig. 3C). The second exception was the PCR assay using the SOPC 10F/10R marker, which had a sensitivity of 100 μg/mL (Fig. 3G). The best result was obtained using a PCR assay with SOPC 3F/3R, suggesting that SOPC 3F/3R may be a reliable diagnostic tool for the detection of fungal pathogens in the field. However, even though the sensitivity of other primer sets, SOPC 1F/1R, SOPC 1-1F/1-1R, SOPC 4F/4R, SOPC 4F/4-1R, SOPD 9F/9R, and SOPD 10F/10R is lower than that of SOPC 3F/3R, these primer sets should be useful for the identification of *P. katsurae* using genomic DNA extracted from pure mycelium *in vitro*.

SCAR markers have been successfully used for the diagnosis and detection of *Phytophthora* species and other fungal pathogens, and have the high sensitivity of PCR [21, 28]. Although the sensitivity of PCR using the SCAR markers in this study was lower than previously reported results, the PCR protocol applied in this study was still applicable, as 100 ng/mL of template DNA can be easily extracted from the pure mycelium of *P. katsurae*, and can produce clear and discernible bands after PCR. Thus, this technique can provide a possible solution for designing specific primers for closely related species.

Detection of *P. katsurae* zoospores. It is important to detect *P. katsurea* zoospores since zoospores can spread chestnut ink disease to non-infected regions within chestnut tree plantations, infecting healthy chestnut trees through the splashing of remaining water in the soil [2]. Seven SCAR markers were applied to detect *P. katsurae* zoospores. The sensitivities of detection of *P. katsurae* zoospores were 10×10^5 zoospores/mL (SOPC 1F/1R), 10×10^5 zoospores/mL (SOPC 1-1F/1-1R), 10×10^1 zoospores/mL (SOPC 3F/3R), 10×10^4 zoospores/mL (SOPC 4F/4R), 10×10^5 zoospores/mL (SOPC 4F/4-1R), and 10×10^4 zoospores/mL (SOPD 9F/9R) (Fig. 4). The best result was obtained using SOPC 3F/3R. In contrast, PCR amplification with SOPD 10F/10R did not amplify any fragments specific to *P. katsurae* (Fig. 4G) suggesting this marker cannot be applied to detect *P. katsurae in vitro* and *in vivo*. In addition, although non-specific bands to *P. katsurae* were produced in this assay, the size and sequence of the amplicon was different from *P. katsurae*. Thus, these primer sets should be a useful tool for specifically detecting *P. katsurae* zoospores without unexpected amplicons from other fungal pathogens. The sensitivity of SCAR markers will likely decrease when these markers are applied to genomic DNA extracted from soil samples and water, as these samples might include substances inhibitory to PCR amplification (e.g., humic acids, lignins, carbohydrates, and resins) [29]. However, alternative strategies, including the addition of BSA, T4g32 proteins,

DMSO, or glycerol to the PCR reaction, can reduce the effects of these PCR inhibitors [30].

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