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Detection of Genus *Phytophthora* and *Phytophthora cryptogea-P. drechsleri* Complex Group Using Polymerase Chain Reaction with Specific Primers

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A technique based on the polymerase chain reaction (PCR) for the specific detection of genus *Phytophthora* and *Phytophthora cryptogea-P. drechsleri* complex group was developed using nucleotide sequence information of ribosomal DNA (rDNA) regions. The internal transcribed spacers (ITS) including 5.8S were sequenced for *P. cryptogea-P. drechsleri* complex group and its related species. Two pairs of oligonucleotide primers were designed. Primer pair ITS1/Phy amplified ca. 240 bp fragment in 12 out of 13 species of *Phytophthora*, but not in *Pythium* spp., *Fusarium* spp. and *Rhizoctonia solani*. Primer pair rPhy/Pcd amplified 549 bp fragment only in *P. cryptogea-P. drechsleri* complex group, but not in other *Phytophthora* spp. and other genera. Specific PCR amplification using the primers was successful in detecting *Phytophthora* and *P. cryptogea-P. drechsleri* complex group in diseased plants.

Keywords **:** *Phytophthora*, *Phytophthora cryptogea-P. drechsleri* complex group, PCR detection, specific primer, ribosomal DNA.

The genus *Phytophthora* is one of the most important plant pathogens attacking almost all plant groups. Current routine methods for the detection and identification of *Phytophthora* species involve using selective media, baiting from soil and isolation from infected plant tissue, followed by pure-culturing of the organism and identification based on morphological characteristics (Erwin and Ribeiro, 1996). In order to improve efficiency and accuracy for the detection of *Phytophthora*, alternative methods such as enzyme immuno-assays (Devergne et al., 1994) and dipstick immuno-assays have been developed (Cahill and Hardham, 1994).

DNA based assays have also been described. Oligonucleotide DNA probes were used for specific detection of *Phytophthora parasitica* and *P. citrophthora* by Goodwin et al. (1990a; 1990b) and of genus *Phytophthora, P. capsici, P. cinnamomi, P. megakarya* and *P. palmivora* by Lee et al. (1993). Detection based on the specific amplification of a target DNA sequence by polymerase chain reaction (PCR) has also been described for *Phytophthora*. Ersek et al. (1994) designed specific primers for *P. parasitica* and *P. citrophthora*. Tooley et al. (1997) for *P. infestans*, *P. erythroseptica* and *P. nicotianae*, Ristaino et al. (1998) for *P. capsici*, and Liew et al. (1998) for *P. medicaginis*. Except Ersek et al. (1994), they all used oligo-nucleotide sequence of ribosomal DNA (rDNA) as a specific primer.

Species complexes, *Phytophthora cryptogea/P. drechsleri* and *P. megasperma* were known as morphological species containing highly divergent genetic groups (Forster et al., 1995). Genetic variation on Korean isolates of *Phytophthora cryptogea/P. drechsleri* species complex were examined (Hong et al., 1998, 1999). The authors reported that there were five genetic groups within thirty Korean isolates of the species complex, and four groups except one originated from cucurbits clustered within a complex group which was named as *P. cryptogea-P. drechsleri* complex group.

In this paper, we described the development of PCR primers derived from the internal transcribed spacer (ITS) region of the rDNA repeats for the specific detection of the genus *Phytophthora* and *P. cryptogea-P. drechsleri* complex group (Hong et al., 1999) in plants. The specificity and absence of cross reactivity were tested on a wide range of *Phytophthora* species and representatives of other fungal genera. Finally, this PCR method was tested for the capacity to detect *Phytophthora* and *P. cryptogea-P. drechsleri* complex group from artificially and naturally infected host tissues.

Materials and Methods

Fungal isolates and genomic DNA extraction. Isolates of *Phytophthora* and other genera used in this study are listed in Table 1. Most isolates were isolated in Korea by Plant Pathology Division, NIAST. All isolates of *Phytophthora* were grown on V8 juice agar and isolates of the other genera were grown on PDA at 25°C.

Extraction of genomic DNA was conducted according to the procedure described in Hong et al. (1999) which basically followed the method of Lee and Taylor (1990).

Amplification of the ITS of rDNA. Primers, ITS1 (5'-TCC-

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Table 1. Fungal isolates used in this study and results of PCR amplification with 3 primer pairs

No.	Species[a]	Isolate no.	Host	PCR products		
				ITS1/ITS4	ITS1/Phy	rPhy/Pcd
1	*Phytophthora cactorum*	KACC 40174[c]	*Pyrus serotina*	+	-	-
2	*P. boehmeriae*	KACC 40173	*Ailanthus altissima*	+	+	-
3	*P. capsici*	KACC 40157	*Capsicum annuum*	+	+	-
4	*P. citrophthora*	KACC 40188	*Malus pumila var dulcissima*	+	+	-
5	*P. nicotianae*	KACC 40163	*Citrus junos*	+	+	-
6	*P. palmivora*	KACC 40167	*Chrysalidocarpus lutescens*	+	+	-
7	*P. citricola*	KACC 40184	*Zizyphus jujuba* var. *inermis*	+	+	-
8	*P. infestans*	KACC 40706	*Lycopersicon esculentum*	+	+	-
9	*P. cambivora*	KACC 40159	*Malus pumila* var. *dulcissima*	+	+	-
10	*P. cinnamomi*	KACC 40182	*Larix leptolepis*	+	+	-
11	*P. cryptogea-P. drechsleri* complex (*P. drechsleri*)[b]	KACC 40190	*Lycopersicon esculentum*	+	+	+
12	〃 (*P. drechsleri*)	KACC 40191	*Angelica gigas*	+	+	+
13	〃 (*P. cryptogea*)	KACC F43	*Gerbera jamesonii*	+	+	+
14	〃 (*P. cryptogea*)	KACC 40413	*Brassica campestris*	+	+	+
15	〃 (*P. megasperma*)	KACC 40401	*Lycopersicon esculentum*	+	+	+
16	*P. melonis* (*P. drechsleri*)	KACC 40194	*Cucumis melo*	+	+	-
17	*P. erythroseptica*	KACC 40200	*Pueraria lobata*	+	+	-
18	*Pythium splendens*	KACC F39	unknown	+	-	-
19	*P. ultimum*	KACC F38	unknown	+	-	-
20	*P. debaryanum*	KACC F40	unknown	+	-	-
21	*P. aphanidermatum*	KACC 40156	*Zoysia japonica*	+	-	-
22	*Rhizoctonia solani* AG-1(IB)	KACC 40111	*Cucumis sativus*	+	-	-
23	*R. solani* AG-2-1	KACC 40124	*Raphanus sativus*	+	-	-
24	*R. solani* AG-4	KACC 40140	*Cucumis sativus*	+	-	-
25	*R. solani* AG-4	KACC 40141	*Capsicum annuum*	+	-	-
26	*Fusarium oxysporum* f. sp. *lycopersici*	KACC 40046	*Lycopersicon esculentum*	+	-	-
27	*Fusarium* sp.	KACC 40241	*Solanum melongena*	+	-	-
28	*F. solani*	KACC 40384	unknown	+	-	-
29	*F. oxysporum* f. sp. *lycopersici*	KACC 40526	*Lycopersicon esculentum*	+	-	-
30	*P. cryptogea-P. drechsleri* complex (*P. drechsleri*)	KACC 40463	*Lactuca sativa*	+	+	+
31	〃 (〃)	KACC 40464	*Spinacia oleracea*	+	+	+
32	〃 (〃)	KACC 40484	*Angelica gigas*	+	+	+
33	〃 (〃)	KACC 40196	*Ligularia fischeri*	+	+	+
34	〃 (〃)	KACC 40465	*Rehmannia glutinosa*	+	+	+
35	〃 (〃)	KACC 40466	*Maximowiczia chinensis*	+	+	+
36	〃 (〃)	KACC 40467	*Lycium chinense*	+	+	+
37	〃 (〃)	KACC 40195	*Atractylodes japonica*	+	+	+
38	〃 (〃)	KACC 40199	*Actinidia chinensis*	+	+	+
39	〃 (*P. megasperma*)	KACC F3020	*Lycopersicon esculentum*	+	+	+
40	*P. melonis* (*P. drechsleri*)	KACC 40193	*Cucumis sativus*	+	+	-
41	〃 (〃)	KACC 40485	*Cucumis sativus*	+	+	-
42	〃 (〃)	KACC 40486	*Cucumis melo*	+	+	-
43	〃 (〃)	KACC 40192	*Cucumis melo*	+	+	-
44	〃 (〃)	KACC 40487	*Cucumis melo*	+	+	-
45	〃 (〃)	KACC 40197	*Citrullus lanatus*	+	+	-
46	〃 (〃)	KACC 40488	*Citrullus lanatus*	+	+	-
47	〃 (*P. melonis*)	KACC 40444	*Cucumis melo*	+	+	-
48	*P. cryptogea-P. drechsleri* complex (*P. cryptogea*)	KACC 40469	*Gerbera jamesonii*	+	+	+

Table 1. Continued

No.	Species[a]	Isolate no.	Host	PCR products		
				ITS1/ITS4	ITS1/Phy	rPhy/Pcd
49	*P. cryptogea-P. drechsleri* complex (*P. cryptogea*)	KACC 40161	*Gerbera jamesonii*	+	+	+
50	〃 (〃)	KACC F44	*Gerbera jamesonii*	+	+	+
51	〃 (〃)	KACC F45	*Gerbera jamesonii*	+	+	+
52	〃 (〃)	KACC 40162	*Gerbera jamesonii*	+	+	+
53	〃 (〃)	KACC 40189	*Brassica campestris*	+	+	+

[a] Species concept by band types generated by PCR-RFLP analysis of ITS and SSU in ribosomal DNA (Hong et al., 1999).
[b] Species name in the blank was determined by morphological and cultural characteristics by Hyeong-Jin Jee, Division of Plant Pathology in NIAST.
[c] Accession number of Korean Agricultural Culture Collection (KACC), Molecular Genetics Division, National Institute of Agricultural Science and Technology, Suwon, Korea.

GTA-GGT-GAA-CCG-CGG-3') and ITS4 (5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3'), designed by White et al. (1990), were used for the amplification of ITS of rDNA. PCRs were conducted in 100 μl reaction volumes. Each reaction tubes contained 1 μl of template DNA solution (≒ 100 ng), prepared above, 10 μl of 10X buffer [50 mM KCl, 100 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 15 mM $MgCl_2$], 4 μl of 2.5 mM (each) dNTP, 0.75 μl (each) of 100 μM primers, 0.5 μl of *Taq* polymerase (5 unit/μl) and 83 μl of ddH_2O. Two drops of mineral oil was placed on the top of each reaction mixture. The thermal cycling parameter was denaturation, 1 min at 95°C, annealing, 1 min at 58°C, and polymerization, 2 min at 72°C. Thirty-five cycles were conducted and the first denaturation and the last polymerization time were extended to 4 min and 8 min, respectively. The success of amplification was monitored by 1% agarose gel electrophoresis.

Cloning and sequencing of the ITS regions. ITS regions of four isolates of *P. cryptogea-P. drechsleri* complex group and five isolates of *P. cactorum, P. cambivora, P. cinnamomi, P. erythroseptica* and *P. melonis* were sequenced to design specific primers. The PCR products were electrophoresed on a 0.8% agarose gel, and then the ITS regions were purified with a QIAquick gel extraction kit (Qiagen GmbH). Purified DNAs were ligated into pGEM-T easy vector (Promega Co.). Ligated plasmids were then transformed into *Escherichia coli* DH5αF' cells, and transformants were selected by blue-white screening procedure (Sambrook et al. 1989). Plasmids containing the ITS regions were isolated by using QIAquick plasmid minikit (Qiagene GmbH). Purified plasmids were sequenced using on an Applied Biosytems 377 automatic sequencer.

Primer design. For specific detection of the genus *Phytophthora*, primer Phy (5'-GCT-ATC-TAG-TTA-AAA-GCA-GA-3') was selected. This oligonucleotide sequence was published by Lee et al. (1993) as oligonucleotide probe for detection of genus *Phytophthora*. Fungal universal primer, ITS1, was used as the pair of the primer, Phy.

For the selection of nucleotide sequence specific for *P. cryptogea-P. drechsleri* complex group, the ITS sequences of the nine isolates were aligned with published sequences from 6 other *Phytophthora* species, using the sequence alignment program CLUSTAL W (Thompson et al., 1994). Regions of dissimilarity in the ITS were used to design a primer specific for *P. cryptogea-P. drechsleri* complex group. Four primers were designed, and the primer, Pcd (5'-CAG-CTT-GCG-CGA-GAA-CAG-AC-3') was selected because it has the highest specificity. Reverse oligonucleotide sequence of Phy, rPhy (5'-TCT-GCT-TTT-AAC-TAG-ATA-GC-3'), was used as the pair of the primer, Pcd.

Test of primer specificity. Specificity of the PCR for the genus *Phytophthora* using primers ITS1/Phy and for the *P. cryptogea-P. drechsleri* complex group using rPhy/Pcd was tested on a collection of seventeen isolates of thirteen *Phytophthora* spp., four isolates of *Pythium* spp., four isolates of *Fusarium* spp. and four isolates of *Rhizoctonia solani* (Table 1). The latter three genera were selected in that they are soil borne fungi as *Phytophthora*, and show similar symptoms with *Phytophthora*. In order to test for the ability to detect other isolates of *P. cryptogea-P. drechsleri* complex group, additional fifteen isolates were checked for the positive amplification. We also tested on nine isolates of *P. melonis* which show similar morphological characteristics with the complex group. PCR parameters for amplification of the specific fragments were identical with those for amplification of ITS region, described above.

Detection of *Phytophthora* in plants. Cucumber stem inoculated with *P. melonis*, Lettuce roots naturally infected by *P. cryptogea-P. drechsleri* complex group from hydrophonics and healthy lettuce roots were used for PCR based detection of *Phytophthora* in plant, *P. cryptogea-P. drechsleri* complex group in plant and negative control, respectively.

Isolation of total DNA from plants basically followed the method of Volossiouk et al (1995). Plant sample (0.5 g) was ground with liquid nitrogen by using a mortar and pestle until fine powder remains. The powdered sample was suspended in 0.5 ml of 0.4% skim milk solution by vigorous vortexing. The debris were removed by centrifugation at 4°C (12,000 × g, 10 min), and the supernatant was mixed with 2 ml of extraction buffer [0.3% SDS in 0.14 M NaCl, 50 mM sodium acetate (pH 5.1)] by vortexing. An equal volume of water saturated phenol solution was added, the phase were mixed by intermittently vortexing for 2 min at room temperature and then separated by centrifugation (12,000 × g, 10 min). The nucleic acid in the aqueous phase was precipitated with 2.5 volumes of ethanol at -80°C for 15 min. The precipitation was collected by centrifugation at 4°C, and the pellet was washed with 70% ethanol, vacuum dried, and then dissolved

A

Scientific name	Isolate no.	GenBank accession no.	Nucleotide sequence (Primer Phy)
			AAGTCTCTGC TTTTAACTAG ATAGCAACTT
Phytophthora capsici	IMI P255	@	
P. cactorum	KACC 40174	AF087480	*****CT*** ********** **********
P. palmivora	IMI P488	@	********** ********** **********
P. citrophthora		@	********** ********** **********
P. citricola	IMI 313729	Y08658	********** ********** **********
P. nicotianae	IMI 208688	Y08673	********** ********** **********
P. infestans		AF007019	********** ********** **********
P. cryp.-P. drech.	KACC 40190	AF087471	********** ********** **********
P. cinnamomi	KACC 40182	AF087478	********** ********** **********
P. melonis	KACC 40193	AF087473	********** ********** **********
P. erythroseptica	KACC 40449	AF087474	********** ********** **********
P. cambivora	KACC 40159	AF087479	********** ********** **********
Pythium aphanidermatum		AF016501	****T*A*G* ******-*CT ***A******
Pythium ultimum		AF016500	*****CT*** *****-**** ***A******
Fusarium oxysporum		X93902	G****AAAC* *T--AA*TA* AT*AA*****
Fusarium solani		L36634	G***A*AACA AGC-**A**A **TAA*****
Rhizoctonia solani		AJ000202	**T*GA*GTA ACGC*T*TA- ATA*TAA*TT

B

			Primer Pcd
			GTCTG TTCTCGC-G- CAAGCTG
P. c-d(P. drechsleri)#	KACC 40190	AF087471	AGGGTGTCTG TTCTGGC-G- TAAGCTGG-G
P. c-d(P. drechsleri)#	KACC 40467	AF087472	********** ***C***-*- C********-*
P. c-d(P. cryptogea)#	KACC 40162	AF087477	********** ***C***-*- C********-*
P. c-d(P. cryptogea)#	KACC 40189	AF087475	********** ***C***-*- C********-*
P. capsici	IMI P255	@	**A***G*G* C*TC***T-- -GTCGA**--
P. cactorum	KACC 40174	AF087480	**A***G*G* C*TC***T-- -GTCGA**--
P. palmivora	IMI P488	@	**A***G*G* C*TC***T-- -GTCGA**--
P. citrophthora		@	**A***G*G* C*TC***T-- -GTCGA**--
P. citricola	IMI 313729	Y08659	**A***G*G* C*TC***T-- -GTCGA**--
P. nicotianae	IMI 208688	Y08674	******G*A* C*TC**TT-- -GTCGA**--
P. infestans		Y08670	**A***G*G* C*TC***T-- -GCCGA**--
P. cinnamomi	KACC 40182	AF087478	******G*G* C*TC***T-- -GTCGA**--
P. melonis	KACC 40193	AF087473	**A***G*G* C*TC***T*T CG**GG-TC*
P. erythroseptica	KACC 40449	AF087474	******G*G* C*TC***T*T CG**GG-TC*
P. cambivora	KACC 40159	AF087479	******G*G* C*TC***T*T CG**GG*TC*

Fig. 1. Nucleotide sequences comparison of (A) *Phyophthora* spp. and related genera in primer Phy region and of (B) *Phytophthora cryptogea-P. drechsleri* complex group and other *Phytophthora* spp. in primer Pcd region. @. The nucleotide sequences are obtained from Cooke and Duncan (1997). #. *P. c-d* ( ): *Phytophthora cryptogea-P. drechsleri* complex group. The species in the blank were identified based on morphological and cultural characteristics by Hyeong-Jin Jee, NIAST.

in 50 μl of water

PCR amplification for detection of *Phytophthora* from DNA extracts of infected plants was also conducted according to the parameter described above.

Nucleotide sequence accession numbers. The complete ITS sequences of the *Phytophthora* spp. determined in this study have been submitted to the GenBank at the NCBI. The accession numbers were shown in Fig. 1.

Results

Nucleotide sequence of ITS from *Phytophthora* and alignment of the sequence in the primer region. Sequencing of the ITS regions from nine isolates of *Phytophthora* revealed that ITS regions of *Phytophthora* spp. were 881-920 bp (GenBank acession no. in Fig. 1), and four isolates of *P. cryptogea-P. drechsleri* complex group were 887-888 bp in size (Fig. 2). Alignment of the ITS sequences showed that sequences of primer Phy region were the same among *Phytophthora* spp. except *P. cactorum*. but the primer sequence had less than 80% homology with *Pythium* spp. and less than 50% homology with *Rhizoctonia solani* and *Fusarium* spp. (Fig. 1A). In primer Pcd, designed for specific detection of *P. cryptogea-P. drechsleri* complex group, the nucleotide sequence showed less than 50% homology with other *Phytophthora* spp. (Fig. 1B). The primers, Phy and Pcd, located at 218 bp and 747 bp from start point of primer ITS1, respectively (Fig. 2).

Specificity of PCR amplification. Primer pair, ITS1/Phy amplified 220-290 bp fragments from isolates of *Phytophthora* spp. (Fig. 3A). The figure shows the absence of cross reactivity of the PCR amplification with the other genera, *Pythium, Fusarium*, and *Rhizoctonia*. However, the fragment was not amplified in *P. cactorum*. Two bases on 3' end of the Phy in *P. cactorum* were different with the primer sequence (Fig. 1).

Primer pair ITS1/Pcd amplified 767 bp fragments from five isolates of *P. cryptogea- P. drechsleri* complex group. However, the primer pair also amplified the same fragment from *Pythium aphanidermatum* (data not shown). In order

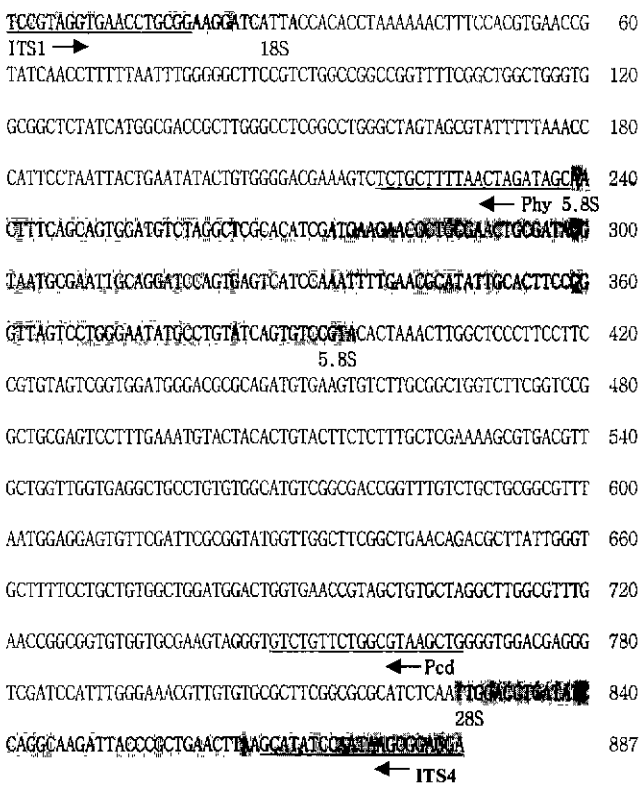

Fig. 2. Nucleotide sequence of the internal transcribed spacer including 5.8S of *Phytophthora cryptogea-P. drechsleri* complex group, isolate KACC 40190. The amplified DNA consists of 3' end of 18S rDNA, ITS I, 5.8S rDNA, ITS II, and 5' end of 28S rDNA. ITS1 and ITS4 are universal primers and Phy and Pcd are selected as primers in this experiment. GenBank accession number of the sequence is AF087471.

to obtain specific amplification only from *Phytophthora*, reverse nucleotide sequence of Phy, rPhy, was used as forward primer instead of ITS1. The primer pair, rPhy/Pcd, amplified 549 bp fragment only from *P. cryptogea- P. drechsleri* complex group (Fig. 3B). Further testing with various isolates of the complex group and *P. melonis* also revealed that the primer pair amplified the fragment only from *P. cryptogea- P. drechsleri* complex group, but not from *P. melonis* which were similar in morphological characteristics (Fig. 3C). The results of PCR amplification with 3 primer pairs were summarized in Table 1.

PCR based detection of *Phytophthora* in plants. Healthy lettuce stem, naturally infected lettuce stem with *P. cryptogea-P. drechsleri* complex group and artificially inoculated cucumber root with *P. melonis* were used as materials for PCR detection of *Phytophthora* from plants. A primer pair, ITS1/ITS4 generated two fragments from infected lettuce stem and cucumber root and one fragment from healthy lettuce stem (Fig. 4). It is believed that ca. 900 bp fragment out of the two bands were originated from *Phytophthora*, and ca. 700 bp fragment out of them from the plants. A primer pair, ITS1/Phy amplified ca. 220 bp fragments only from the *Phytophthora*-diseased plants. A primer pair rPhy/Pcd amplified 549 bp fragments only from *P. cryptogea-P. drechsleri* complex group infected lettuce stem.

Discussion

Sequencing and analysis of the ITS region of *Phytophthora* spp. allowed us to design specific PCR primers. These primer sets, ITS1/Phy and rPhy/Pcd, successfully amplified DNA fragments from *Phytophthora* and *P. cryptogea-P. drechsleri* complex group, respectively. The specificity of the PCR based detection method was verified by the absence of cross reactivity with DNA from *Pythium* spp., *Fusarium* spp., and *Rhizoctonia solani*. This specific PCR amplification was used to detect *Phytophthora* in artificially inoculated cucumber stem and *P. cryptogea-P. drechsleri* complex group in naturally infected lettuce root.

Primer pair, ITS1/Phy could not amplify ca. 220 bp fragment from *P. cactorum* (Fig. 3A). It seems to be strange that the Phy primer did not anneal to rDNA of *P. cactorum*, because its nucleotide sequence differs only in 2 bp out of 20 bp (Fig. 1A). The oligo-nucleotide probe which had the identical nucleotide sequence with Phy primer, was hybridized with rDNA of *P. cactorm* in Lee et al. experiment (1993). However, nucleotide sequence of 3' end of primer is very important for PCR amplification, because extension of nucleotide sequence does not occur if the 3' end does not match to template. In *P. cactorum*, 2 bases at 3' end differ from those of the primer (Fig. 1A). Three to five base extension of the primer sequence or shift of the primer to 5' direction may be a solution, but this change may make the primer anneal to template of *Pythium ultimum*. More research is required for the design of better primer for the specific detection of the genus *Phytophthora*.

The primer pair, rPhy/Pcd amplified ca. 550 bp fragments from *P. cryptogea- P. drechsleri* complex group, but did not from the other 12 *Phytophthora* spp., *Pythium* spp., *Fusarium* spp. and *Rhizoctonia solani* (Fig. 3B). Nucleotide sequence alignment of the primer region showed less than 50% homology between *P. cryptogea-P.drechsleri* complex group and other *Phytophthora* spp. (Fig. 1). Therefore, it could be told that the primer pair had high specificity to detect *P. cryptogea-P. drechsleri* complex group among *Phytophthora* spp.

The primary factors in developing a PCR detection method are sensitivity as well as specificity. Use of designed primers from the rDNA regions has far superior sensitivity compared to the use of random non-defined primers (Liew et al., 1998) because rDNA occurs in multiple copy [up to 200 copies (Bruns et al., 1991)] per haploid genome and is highly stable. Lee and Taylor (1990) reported PCR detec-

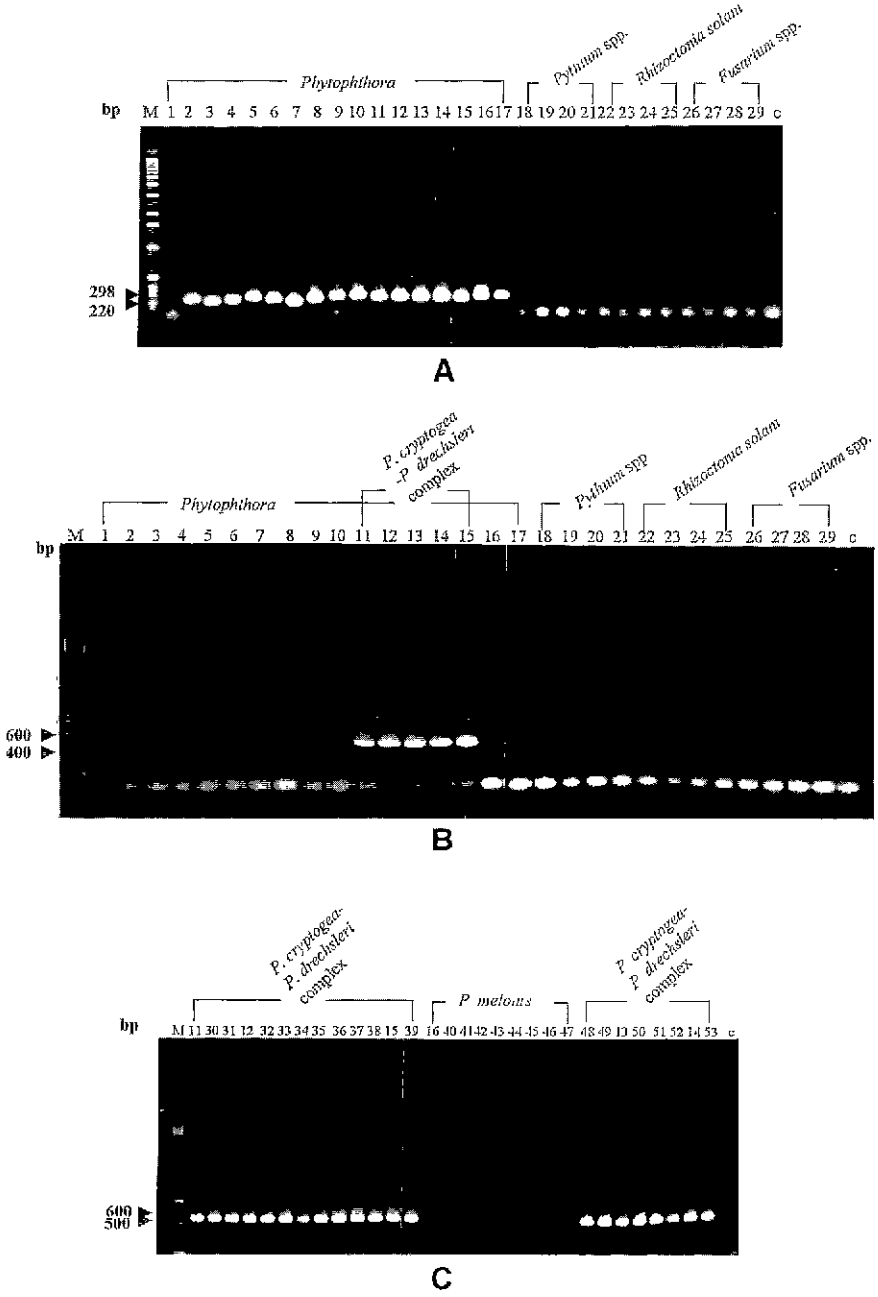

Fig. 3. PCR amplification of various isolates of *Phytophthora, Pythium, Fusarium* and *Rhizoctonia solani* (A) using ITS1/Phy and (B), (C) using rPhy/Pcd as primer pair. The numbers above panels correspond to the numbers of Table 1. c, negative control.

tion of rDNA from a single spore of *Neurospora tetrasperma*, and Liew et al. (1998) reported that primer pair from rDNA enabled PCR amplification from template DNA mixtures with high host-pathogen DNA ratio, as well as from picogram levels of purified genomic DNA. The current primer pairs, ITS1/Phy and rPhy/Pcd, originated from rDNA are also thought to be highly sensitive.

The PCR assay with infected plants can be performed in a day, including sample processing, PCRs, gel electrophoresis and staining. Tooley et al. (1997) reported that the PCR detection of *P. infestans* from potato can be performed in 3 to 4 hours. In the PCR assay, time limiting step is sample processing. The sampling method (Volossiouk et al., 1995) for PCR used in this study are time consuming and are not so efficient. Fast and easy method for sample processing was published (Ristaino et al. 1998; QIAGEN, 1995; Wang et al., 1993; Edwards et al. 1991). Combined with the use of the rapid NaOH method of sample processing (Ristaino et

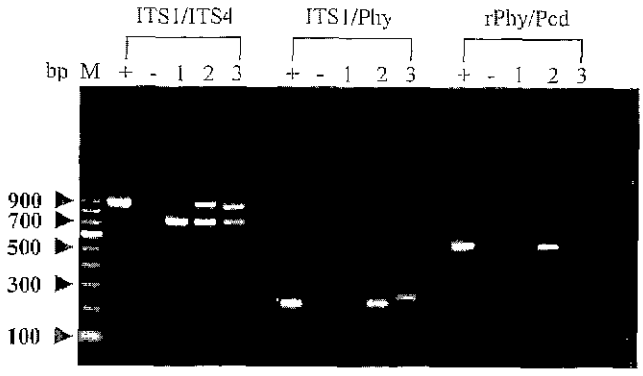

Fig. 4. PCR detection of *Phytophthora* in diseased plants. +, pure genomic DNA of *Phytophthora cryptogea-P. drechsleri* complex group, KACC 40190; −, no template DNA; 1, healthy lettuce root; 2, lettuce root naturally infected by *Phytophthora cryptogea-P. drechsleri* complex group; 3, cucumber stem artificially inoculated with *P. melonis*.

al., 1998), the PCR primers we described will offer a fast and useful method for detecting *Phytophthora* in plants.

PCR detection of *Phytophthora* in plant may be inconsistent. To solve this problem, we recommend PCR amplification with other primer pairs, ITS1/ITS4, as well as ITS1/Phy or rPhy/Pcd (Fig. 4). If the sample processing and PCR amplification are successful, it is believed that ITS fragments of plant are amplified whether the plant is infected with *Phytophthora* or not. Furthermore, size of the ITS region of *Phytophthora* is ca. 900 bp, and is much larger than that of ITS of plants and other fungi (Fig. 4).

The current primer pairs designed from ITS of rDNA were successful in detecting *Phytophthora* from DNA isolated from infected host tissue. The specificity of the primers was verified by the absence of cross reactivity with other genera. A further study will enable the primers and the method to be used in practice for rapid detection of the *Phytophthora* from plant. The current primer pairs may be also used for the detection of the pathogen in soil samples.

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