Selection of RAPD Markers for *Phytophthora infestans* and PCR Detection of *Phytophthora infestans* from Potatoes

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For rapid and secure differentiation of *P. infestans* from other *Phytophthora* species, two fragments obtained from randomly amplified polymorphic DNA (RAPD) profiles were selected as markers. Also, primers for in polymerase chain reaction (PCR) to detect *P. infestans* specifically were developed by analyzing the sequences of ITSII regions in rDNA of *Phytophthora* species. The primers, PISP-1 and ITS3 amplified a single. Fragment 450 bp of about in *P. infestans*, but not in other fungal or bacterial isolates. Annealing temperatures and template DNA quantities were varied for the optimization of PCR conditions. From the result of the PCR detection study, species-specific primers were selected under annealing temperatures ranging from 55°C to 61°C, and template DNA levels ranging from 10 pg to 100 ng.

Key words: ITS regions, molecular markers, *Phytophthora infestans*, potato late blight, RAPD, rDNA

Phytophthora infestans (Mont) de Bary, the causative agent of late blight of potatoes (*Solanum tuberosum* L.) and tomatoes (*Lycopersicon esculentum* Mill), is one of the most important plant pathogens. Populations of *P. infestans* in Central Mexico are highly diverse, indicating that this area is the probable center of origin of the pathogen (8, 9, 12). From this region, the late blight pathogen has probably migrated to other parts of the world via airborne spores or infected potatoes or tomatoes (6, 11).

Identification of most fungi is principally based on morphological characteristics. For several genera of fungi, including *Phytophthora*, accurate identification of species can be very difficult and time-consuming because morphological features overlap among species (7). As a result, Clare (5) used proteins in electrophoresis for differentiating *Pythiaceous* fungi. More recently, isozyme and restriction fragment length polymorphism (RFLP) have been used to differentiate fungi with similar characteristics (27, 30, 31, 33). As well, PCR is a sensitive method that requires very little genomic DNA, thereby saving time and labor (12, 39).

The randomly amplified polymorphic DNA (RAPD) technique has been used as an auxiliary tool for the genetic analysis, classification, or identification of soil-borne pathogens such as *Fusarium, Rhizoctonia,* or *Colletotrichum* (1, 3, 4, 20, 22, 36). In recent years, many researchers have studied internal transcribed spacer (ITS) regions of nuclear

ribosomal DNA (rDNA) to analyze genetic differences and taxonomical relationships among *Phytophthora* species (19), *Pythium* species (2), *Peronosclerospora* species (40), mycorrhizae and rust organisms (10), *Verticillium* species (23) and *Fusarium* species (25). This is because ITS regions are of suitable size for PCR amplification, restriction analysis, and sequencing procedures and because ITS regions are variable among species as well (12, 38).

If *P. infestans*, which can severely decrease potato yields, is rapidly and accurately detected, this would be useful in the prediction and control of late blight, reduction of pesticide costs, harvest of healthy potatoes, and differentiation of *P. infestans* from other *Phytophthora* species. Therefore, our objective in this study was to develop a PCR-based method using RAPD of the ITSII region for more rapid sensitive and accurate species-specific detection of *P. infestans* than previous works (16, 33, 35).

Materials and Methods

Fungal strains and genomic DNA isolation

Isolates, including *P. infestans*, used in this study were either obtained from infected plants or obtained from other researchers (Tables 1 and 2). Isolates of *P. infestans* were cultured in rye medium (60 g/L rye grain, 20 g/L dextrose) for 10 days at 18°C (14). Other *Phytophthora* species and fungal pathogen isolates were cultured in V8 juice (200 ml/L V8 juice, 5 g/L $CaCO_3$) or PDB (20 g/L potato dextrose broth, 20 g/L dextrose) media for 10 days at 24°C in

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Table 1. Isolates of *Phytophthora* species used to generate RAPD profiles

Lane no.	Isolates	*Phytophthora* species	Host plants
1	P-9827	*Phytophthora infestans*	*Lycopersicon esculentum* M_{ILL}.
2	P-9829	"	"
3	HY-1	"	*Solanum tuberosum* L.
4	P-9833	"	*Lycopersicon esculentum* M_{ILL}.
5	P-9834	"	"
6	BF-8	"	"
7	BN-11	"	"
8	BC-18	"	"
9	40401	*P. megasperma*	"
10	40412	*P. sojae*	*Glycine max* M_{ERR}.
11	40174	*P. cactorum*	-
12	40182	*P. cinnamomi*	-
13	40184	*P. citricola*	*Zizyphus jujuba* M_{ILL}.
14	40189	*P. cryptogea*	*Brassica campestris* subsp. *napus* var. *pekinensis* M_{AKINO}.
15	40157	*P. capsici*	*Capsicum annuum* L.
16	96CC7116	"	"
17	96CY1220	"	"
18	96CA8103	"	"
19	97CC7112	"	"
20	97CC7302	"	"
21	KD-1	*Phytophthora sp.*	*Solanum tuberosum* L.

darkness. Bacterial isolates were grown at 27°C overnight in Luria-Bertani broth (29). Genomic DNA of each isolate was extracted using a modification of the method described

by Goodwin *et al.* (12, 13), and long-term stocks were maintained as agar plugs in 10% glycerol under liquid nitrogen (32). The concentrations of RNase-treated DNAs were determined by spectrophotometery or estimated on 0.8% agarose gels stained with ethidium bromide.

PCR conditions for RAPD and selection of species-conserved and specific markers

Based on the modified RAPD protocol of Williams *et al.* (39), PCR reactions (20 µl) contained 10 ng of template DNA, 200 µM each of deoxynucleotide, 1 unit of *Taq* DNA polymerase, reaction buffer, and 0.5 µM of primer. As the RAPD primers used in this study, 10 primers in OPA and OPB series were obtained from Operon Technologies Inc., USA. PCR was performed using PTC-100TM (MJ Research Inc., USA). The reaction conditions consisted of 5 min at 95°C (preheating), 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C. After 45 cycles, the final extension period was carried out for 10 min at 72°C before terminating the PCR at 4°C. PCR products were electrophoresed on 1.5% TBE (0.089 mM Tris, 0.089 mM Boric acid, and 0.02 mM EDTA) agarose gels. In order to confirm polymorphic markers, the amplifications were repeated at least three times.

ITSII amplification and cloning

For PCR amplification of the ITSII region (Fig. 1) of *P. infestans* and other *Phytophthora* species, 10ng of genomic

Table 2. Isolates of *Phytophthora* and other pathogens used for the species-specific detection of *P. infestans*

Lane no.	Isolates	Pathogens	Mating type	Metalaxyl sensitivity	Hosts
1	KPI-05	*Phytophthora infestans*	A1	Sensitive	*Solanum tuberosum* L.
2	KPI-31	"	A1	Moderate	"
3	KPI-11	"	A1	Resistant	"
4	KPI-64	"	A2	Sensitive	"
5	KPI-52	"	A2	Moderate	"
6	KPI-07	"	A2	Resistant	"
7	KD-1	*Phytophthora* sp.			-
8	40401	*P. megasperma*			*Lycopersicon esculentum* MILL.
9	40412	*P. sojae*			*Glycine max* MERR.
10	40174	*P. cactorum*			-
11	40182	*P. cinnamomi*			-
12	40184	*P. citricola*			*Zizyphus jujuba* MILL.
13	40189	*P. cryptogea*			*Brassica campestris* subsp. *napus* var. *pekinensis* MAKINO
14	40157	*P. capsici*			*Capsicum annuum* L.
15	96CC7116	*P. capsici*			"
16	KCT214	*Fusarium oxysporum*			*Cucumis sativus* L.
17	WS5-3	*Fusarium oxysporum*			"
18	R25	*Rhizoctonia solani*			*Solanum tuberosum* L.
19	R28	*Rhizoctonia solani*			"
20	E301629	*Erwinia carotovora* subsp. *atroseptica*			"
21	E301397	*Erwinia carotovora* subsp. *carotovora*			*Capsicum annuum* L.
22	P2108	*Ralstonia solanacearum*			*Solanum tuberosum* L.

Primer
ITS 3: 5'-GCATCGATGAAGAACGCAGC-3'
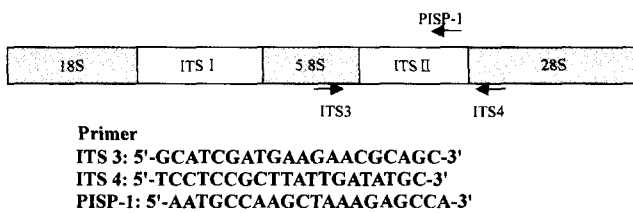ITS 4: 5'-TCCTCCGCTTATTGATATGC-3'
PISP-1: 5'-AATGCCAAGCTAAAGAGCCA-3'

Fig. 1. Genetic map of a portion of the rDNA repeat showing the location of oligonucleotide primer sites used to amplify rDNAs from *Phytophthora* species.

DNA of each isolate was added to 24 µl of reaction mixtures containing 200 µM dNTPs, 0.1 µM each of the primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), 1 unit of *Taq* polymerase (Dynazyme™, USA), 1 × PCR buffer and dH₂O. The reaction conditions consisted of 1 min at 95°C (pre-heating), 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C, and 8 min at 72°C for 30 cycles, followed by storage at 4°C. Reactions were performed in PTC-100™ (MJ Research Inc., USA). Amplified PCR products were identified on 3% agarose (Metaphor agarose) and purified with Wizard PCR Preps (DNA Purification System, USA). The purified DNA fragments were ligated into pGEM®-T Easy Vector (Promega, USA) and used to transform competent cells of *Escherichia coli* strain JM109. Inserted plasmids were extracted with Quanta-Plasmid™ amplification QUKP (Quantum Biotechnologies Inc., USA). Ampicillin-resistant colonies were screened by PUC/M13 forward primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and PUC/M13 reverse primer (5'-TCACACAGGAAACAGCTAT-GAC-3'), or by a restriction of the cloned vectors.

Sequence analysis and primer selection

Thermal cycle sequencing was performed on sequence-grade plasmid DNA with a silver SequenceTM DNA sequencing system (Promega, USA). The sequence data were compared to the DNA sequence data registered on GenBank in the National Center for Biotechnology Information (NCBI). Subsequently, identified sequences were aligned with the DNASTAR Computer software program (DNASTAR Inc., USA). After screening with the software program, primers were tested against various fungal and bacterial genomic DNAs.

Southern blot hybridization

PCR products amplified by ITS3 and PISP-1 primers were electrophoretically separated on 1% agarose TBE gel. After the gels were stained with ethidium bromide, they were transferred to a nylon membrane (Hybond™-N⁺, Amersham, England). This membrane was prehybridized and hybridized in a hybridization incubator (Robbins Scientific, USA). The plasmid to be inserted with the ITSII region of *P. infestans*, KIP-05, was prepared for southern blot analysis by a method of random-primed labeling. A total

of 100 µl of PCR labeling reaction mixture containing 10 ng of template DNA, 0.5 mM of each primer (ITS3 and ITS4), 1 × PCR buffer, 5 unit *Taq* polymerase (Dynazyme, USA), 0.1 mM of dGTP, dCTP, and dATP, 0.09 mM dTTP, and 1 mM digoxigenin-dUTP (Boehringer Mannheim, Germany) was prepared and reacted in a program consisting of 90 sec at 94°C, 90 sec at 50°C, 2 min at 72°C for 24 cycles, and 4 min at 72°C. Detection involved using a DIG luminescent detection kit and all procedures were carried out using the supplied instruction manual (Boehringer Mannheim, Germany).

Investigation of PCR sensitivity

In order to assess the sensitivity of amplification by PCR using the primer set for molecular detection, reactions were performed using annealing temperatures ranging from 55°C to 67°C. Different genomic DNA quantities from 100 ng to 10 pg were also prepared by continual dilutions. The PCR reaction was carried out as described above. PCR products were electrophoretically separated on 1.0% agarose gel, stained with ethidium bromide (10 mg/ml) for 30 min and visualized with UV light.

Inoculation and sampling for PCR assay

Tubers and leaves naturally infected by *P. infestans* were collected and, in addition, potato tubers were artificially inoculated. Agar plugs of V8 medium containing *P. infestans* isolate KPI-05 were placed into wound cuts of tubers sterilized with 6% sodium hypochlorite in advance. The wounds were then sealed with the tuber plug, placed in culture

Table 3. Tuber and leaf tissue samples naturally or artificially infected with *P. infestans* and used for extraction of total DNA

Lane no.	DNA-extracted tissue area
M	Marker No. XVII (Boehringer Mannheim, Germany)
1	no DNA (negative control)
2	uninfected healthy tuber
3	uninfected healthy leaf
4	artificially infected tuber, center of late blight lesion
5	artificially infected tuber, area between the center and margin of late blight lesion
6	artificially infected tuber, margin of late blight lesion
7	artificially infected tuber, tissues next to margin of late blight lesion
8	artificially infected tuber, area of the tuber without symptoms
9	naturally infected tuber, center of late blight lesion
10	naturally infected tuber, area between the center and margin of late blight lesion
11	naturally infected tuber, margin of late blight lesion
12	naturally infected tuber, tissues next to margin of late blight lesion
13	naturally infected tuber, area of the tuber without symptoms
14	naturally infected leaf, necrotic center of late blight lesion
15	naturally infected leaf, sporating zone of late blight lesion
16	naturally infected leaf, water-soaked area of late blight lesion
17	naturally infected leaf, green and healthy zone of late blight lesion
18	crown of the infected plant
19	stem of the infected plant

bottles containing wet filter paper, and incubated at 18°C in darkness for 10~14 days.

To ascertain whether *P. infestans* could be detected directly in potato tissues using PCR, small tissue pieces (5 × 5 mm) were excised from regions of visible necrosis and from healthy regions of tubers and leaves (Table 3). These small pieces were placed into PCR tubes and macerated using a tissue grinder (Fisher, USA). The procedures followed for PCR were based on the method described by Wang *et al.* (37).

Results

Selection of species-conserved and-specific markers from RAPD profiles

Different isolates of *Phytophthora* were analyzed for RAPD profiles with arbitrary primer sets (Table 1). Among the OPA and OPB primer series, we selected two distinct markers generated with the OPA20 (5'-GTTGCGATCC-3'). This primer amplified both a ca. 680 bp fragment specific to *P. infestans* and a 600 bp fragment conserved among *Phytophthora* species (Fig. 2).

ITSII amplification and cloning

ITSII regions between 5.8S and 28S for all *Phytophthora* species were amplified with ITS3 and ITS4 primers (Fig. 1). A single band of ca. 650 bp was obtained from all *Phytophthora* species (data not shown). Among *Phytophthora* species, we chose isolates KPI-05, KPI-11, KPI-31, KPI-52, KPI-64, 40157, 40401, and 97CC7112 (Table 2) for the insertion of ITSII regions into vectors.

Sequence analysis, primer selection and southern hybridization

Cloned isolates, KPI-05, KPI-31, KPI-11, KPI-64, KPI-52, 40401, 40157, and 97CC7112 (Table 2) were used for the analysis of nucleotide sequences through manual sequencing. As shown in Fig. 3, ITSII of KPI-05 consisted of 556 nucleotides. The sequences were compared with those available in the Genbank sequence database (Accession number: PINITS2) and ribosomal internal transcribed spacer II of *P. infestans* showed 98% homol-
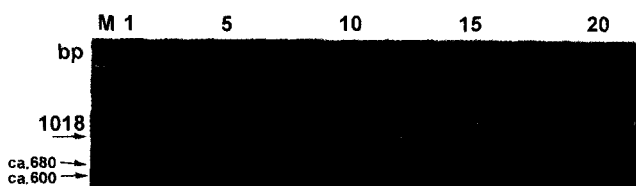

Fig. 2. RAPD profile generated with OPA20 (5'-GTTGCGATCC-3'). Lane M is DNA molecular weight markerX (Boehringer Mannheim, Germany) and numbers on top of the lane indicate the isolate number of each species, as shown in Table 1.

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        10        20        30        40
   |||||||||||||||||||||||||||||||||||||||
TCCTCCGCTTATGATATGCTTAAGTTCAGCGGGTAATCTT 40
GCCTGATATCAGGTCAATTGAGATGCATACATACCACAAC 80
AATTTCCCAAATTGGATCGGACCCTCGGCAGCCGAAGCCG 120
CCACTCTACTTCGCAACAGCAAAGCCGATTCAAATGCCAA 160
GCTAAAGAGCCATGGTTCACCAGTCCATCACGCCACAGCA 200
       210       220       230       240
   |||||||||||||||||||||||||||||||||||||||
GGAAAATCACCCAATAAGCGGCATTGTTCAGCCGAAGCCA 240
ACCATACCACGAATCGAGCATTTCTCCATTAACGCCGCAG 280
CAGACAAACGGGTCGCCAACTCGCTACAATAGCACCGTCC 320
ACAACCAGCAATGCCACCACTTTTGGAGCAAAGAGAAGTA 360
CAGTTTAGTACATTTAAAAGGACTCGCAGTSGGTCCGAAA 400
       410       420       430       440
   |||||||||||||||||||||||||||||||||||||||
ACCAACCGCAAGACACTTCACATCTGGCATCTCCTCCACC 440
GACTACACGGAAGGAAGAAAGCCAAGTTTGTTGTACGGAC 480
ACTGATACAGGCATACTTCCAGGACTAACCCGGAAGTGCA 520
ATATGCGTTCAAAATTTCGATGACTCACTGAATCCTGCAA 560
TTCGGCAATTBWCGCAGTTC 580
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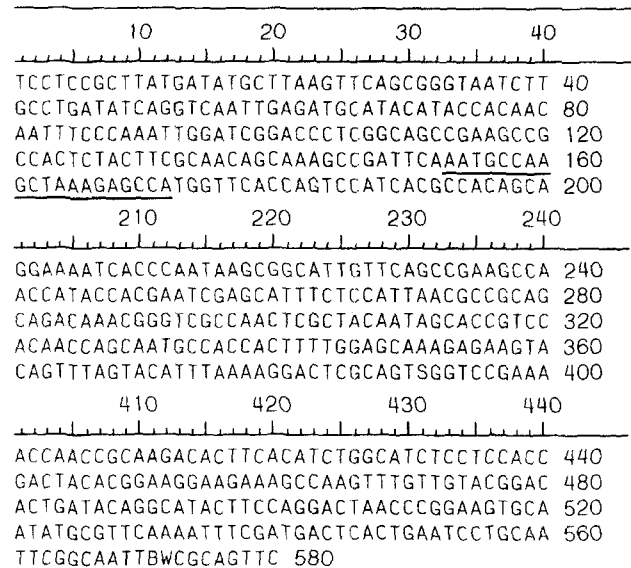
Fig. 3. The complete sequence of the ITSII region of *Phytophthora infestans*, KPI-05. PISP-1 primer is underlined.
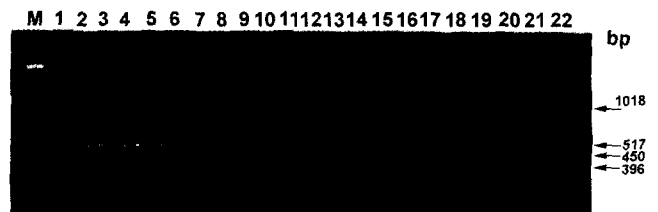

Fig. 4. The amplification of a 450 bp product using primer pairs, PISP-1 and ITS 3, to detect *P. infestans* specifically. Lanes M; 1kb DNA ladder, 1~6; KPI-05, KPI-31, KPI-11, KPI-64, KPI-52, and KPI-07, 7; KD-1, 8; 40401, 9; 40412, 10; 40174, 11; 40182, 12; 40184, 13; 40189, 14; 40157, 15; 96CC7116, 16; KCT214, 17; WS5-3, 18; R25, 19; R28, 20; E301629, 21; E301397, and 22; P2108 (Table 2).

ogy (data not shown). Sequences of ITSII were aligned with DNASTAR program (DNASTAR Inc., USA) to design proper primers that could amplify *P. infestans* specifically. We chose one of the several primers, designated as PISP-1 (5'-AATGCCAAGCTAAAGAGCCA-3') for use in further experiments. The primers, ITS 3 and PISP-1, were used in a PCR mixture to obtain a 453 bp amplified fragment in *P. infestans* only. Therefore, ITS3 and PISP-1 primers were found to identify and detect *P. infestans* specifically (Fig. 4). However, the primers did not amplify DNA of other fungi and bacteria. A probe encoding the ITSII region of isolate KPI-05 was hybridized with PCR products amplified by ITS3 and PISP-1 primers. In southern hybridization (Fig. 5), we observed the same results as with the primer pairs.

Sensitivity of the primer set for molecular detection

The above two 20 bp oligonucleotide primers for specific detection of *P. infestans* were used to investigate annealing temperature ranges and detectable genomic DNA con-
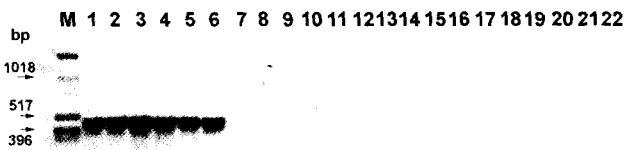
Fig. 5. The southern hybridization with labeled plasmid pGEM® T-easy vector containing the cloned ITSII region from *P. infestans* isolate KPI-05. Lanes M; 1kb DNA ladder, 1~6; KPI-05, KPI-31, KPI-11, KPI-64, KPI-52, and KPI-07, 7; KD-1, 8; 40401, 9; 40412, 10; 40174, 11; 40182, 12; 40184, 13; 40189, 14; 40157, 15; 96CC7116, 16; KCT214, 17; WS5-3, 18; R25, 19; R28, 20; E301629, 21; E301397, and 22; P2108 (Table 2).
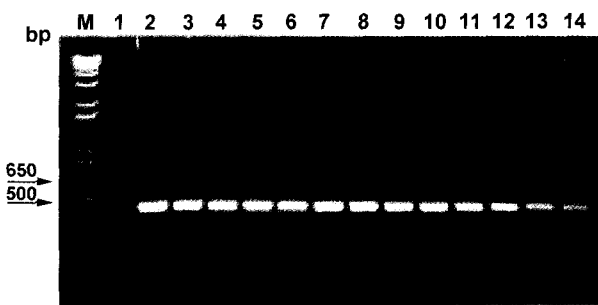

Fig. 6. Primer sensitivity according to template DNA quantities of *P. infestans* determined by PCR using PISP-1 and ITS3 primers. PCR products were resolved by 1% agarose gel electrophoresis. Lane M; 1 Kb PLUS Ladder (Gibco, USA), 1; no DNA (negative control), 2; 100 ng, 3; 10 ng, 4; 1 ng, 5; 800 pg, 6; 600 pg, 7; 400 pg, 8; 200 pg, 9; 100 pg, 10; 80 pg, 11; 60 pg, 12; 40 pg, 13; 20 pg, and 14; 10 pg.

centrations. The annealing temperature was a crucial factor in optimizing product formation. Although produced yields were different, they were detectable at the range of 55°C to 61°C on stained agarose gels (data not shown). In addition, when used to elucidate genomic DNA quantity, these primer sets amplified a visible segment at 10 pg~100 ng template DNA levels (Fig. 6).

Detection of P. infestans in infected potato tissues

Template genomic DNA was extracted from different plant tissue areas using the method of Wang *et al.* (37). No PCR products were amplified from uninfected tubers, leaves or the control, but there was a single band (ca. 450 bp) amplified from artificially and naturally infected tubers, as well as from naturally infected leaves (Fig. 7). In the tuber assay, *P. infestans* was detected in dark, sunken lesions as well as healthy tuber tissue. DNA from healthy leaves (lane 17 in Fig. 7) and crown regions (lane 18 in Fig. 7) was not amplified. DNA from artificially infected tubers with no symptoms (lane 8 in Fig. 7) was amplified because the tissues were already invaded with the fungus even without symptoms were not shown. DNA from naturally infected water-soaked leaf lesions (lane 16 in Fig.

Fig. 7. Detection of *P. infestans* in different tissues (Table 3), using PISP-1 and ITS3 primers. Lane M; marker No. XVII (Boehringer Mannheim, Germany), 1) no DNA (negative control), 2) uninfected tuber, 3) uninfected leaf, 4~8) artificially infected tuber, 9~13) naturally infected tuber, 14) naturally infected leaf, necrotic center of late blight lesions, 15) naturally infected leaf, sporating zone of late blight lesion , 16) naturally infected leaf, water-soaked area of late blight lesion, 17) naturally infected leaf, green and healthy zone of late blight lesion, 18) crown of the infected plant without symptoms, and 19) stem of the infected plant without symptoms.

7) were not amplified due to the lack of fungal invasions in water-soaked regions.

Discussion

DNA-based diagnostic methods have been developed as highly sensitive and species-specific tools, as described previously (17, 18). These techniques are very powerful for the detection and taxonomy of fungi. PCR-based techniques, especially, are much more valuable than conventional methods because they require small quantities of DNA and little time. They are also usually done together with negative controls.

In this work, we selected two fragments, one found in all *Phytophthora* species and the other specific to *P. infestans* only, from RAPD profiles of *Phytophthora* species in South Korea using the OPA20 primer of 10 primers in the OPB and OPA series. In general, most arbitrary primers produced complex banding patterns that were difficult to interpret and the patterns were not always reproducible. However, the two selected major fragments gave the same results in three repeated experiments. Further research to develop sequence-characterized RAPD fragments (SCARs), which was first applied by Paran and Michelmore (28) to generate specific PCR markers using the ca. 680 bp of distinct segments, is one way of developing the specific markers.

Previously, Tooley *et al.* (34) developed the specific primer, PINF, and effectively detected *P. infestans* only. Trout *et al.* (35) evaluated the accuracy of the PINF primer using many *Phytophthora* species. *Phytophthora cactorum* and *P. mirabilis* were detected with PINF primer, and these two species could be further differentiated by digesting the PCR products with some restriction enzymes (16, 35). We have developed another species-specific primer set: ITS3 and PISP-1. The PISP-1 primer was developed according to stringent standards (24) for designating effectual primers and through sequence alignment. It could amplify

only the specific PCR-band for *P. infestans*. Although *P. infestans* populations may contain sexual compatible types and isolates with different metalaxyl sensitivities, they all were detectable. This was especially the case with *P. cactorum*, which could be directly differentiated from *P. infestans* without treatment of amplified products with restriction enzymes (Fig. 4). We have ascertained that PISP-1 could not amplify any bands of *P. nicotianae* and *P. erythroseptica* among pathogens of potatoes in additional work.

The 453 bp specific fragment size, excepting primer oligonucleotides, is within the range suggested to be optimal for amplification by PCR (15, 26). We also investigated the annealing temperature and template DNA quantity, since they are factors that could influence the rate and specificity of amplification (15, 21, 26). The theoretical annealing temperature calculated from primers was 68°C (data not shown). The temperatures of 55°C~61°C were suitable for the observation of PCR products of *P. infestans* on stained agaroses. These values were far below the theoretical optimal temperature of 68°C. Ten pg~100 ng of *P. infestans* genomic DNA was sufficient for detectable amplification by PCR (Fig. 6).

In the detection tests of infected leaf or tuber, no PCR products were amplified from uninfected tubers, leaves or the control, but there was a single band (ca. 450 bp) amplified from artificially and naturally infected tubers, as well as from naturally infected leaves (Fig. 7). In the tuber assay, *P. infestans* was detected in dark, sunken lesions as well as healthy tuber tissue. DNA from healthy leaves (lane 17 in Fig. 7) and crown regions (lane 18 in Fig. 7) was not amplified. DNA from artificially infected tubers with no symptoms (lane 8 in Fig. 7) was amplified because the tissues were already invaded with the fungus even without symptoms were not shown. DNA from naturally infected water-soaked leaf lesions (lane 16 in Fig. 7) were not amplified due to the lack of fungal invasions in water-soaked regions. The applification of PCR technology effectively allows detection of organisms *in vitro* and *in planta* and can provide taxonomic data. Therefore, further work such as the *in planta* detection of *P. infestans* in infected tissues or tubers of potatoes, and at different developmental stages of the host *in vivo* and *in vitro*, are possible.

Acknowledgments

References

1. Angela G.S., M.M. Evelyn, and H.G. Hartwig. 1996. Polymerase chain reaction-based assays for species-specific detec-

tion of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. *Phytopathology* 86, 515-522.

2. Chen, W. 1992. Restriction fragment length polymorphisms in enzymatically amplified ribosomal DNAs of three heterothallic *Pythium* species. *Phytopathology* 82, 1467-1472.

3. Choi, H.S., K.S. Kim, M.J. Kim, and Y.S. Lee. 1997. RAPD analysis for the evaluation of genetic diversity among the *Fusarium* species from various sources. *Korean J. Mycology* 25, 202-208.

4. Choi, H.S., K.S. Kim, and Y.S. Lee. 1997. Molecular analysis of genetic diversity among the *Fusarium oxysporum* and their forma specialis from various sources. *J. Agricultural Science* 8, 29-36.

5. Clare, B.G. 1963. Starch-gel electrophoresis of protein as an aid in identifying fungi. *Nature* 200, 803-804.

6. Drenth. A., S.B. Goodwin, W.E. Fry, and L.C. Davidse. 1993. Genotypic diversity of *Phytophthora infestans* in the Netherlands revealed by DNA polymorphism. *Phytopathology* 83, 1087-1092.

7. Erwin, D.C. 1983. Variability within and among species of *Phytophthora*. p. 149-165. *In* D.C. Erwin, S. Bartnicki-Garcia, and P.H. Tsao (eds.), Phytophthora: its biology, taxonomy, ecology and pathology. American Phytopathological Society, St. Paul, Minn. USA.

8. Fry, W.E., S.B. Goodwin, J.M. Matuazak, L.J. Spielman, and M.G. Milgroom. 1992. Population genetic and international migrations of *Phytophthora infestans*. *Annu. Rev. Phytopathol.* 30, 107-129.

9. Gallegly, M.E. and J. Galindo. 1958. Mating types and oospores of *Phytophthora infestans* in nature. *Phytopathology* 48, 274-277.

10. Gardes, M. and T.D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2, 113-118.

11. Goodwin. S.B. 1997. The population genetics of *Phytophthora*. *Phytopathology* 87, 462-473.

12. Goodwin, S.B., A. Drenth, and W.E. Fry. 1992. Cloning and genetic analysis of two highly polymorphic moderately repetitive nuclear DNAs from *Phytophthora infestans*. *Curr. Genet.* 22, 107-155.

13. Goodwin, S.B., L.J. Spielman, J.M. Matuszak, S.N. Bergeron, and W.E. Fry. 1992. Clonal diversity and genetic differentiation of *Phytophthora infestans* population in Northern and Central Mexico. *Phytopathology* 82, 955-961.

14. Griffith, G.W., R. Snell, and D.S. Shaw. 1995. Late blight (*Phytophthora infestans*) on tomato in the tropics. *Mycologist* 9, 87-89.

15. Innis, M.A. and D.H. Gelfand. 1990. Optimization of PCRs. p. 3-12. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.), PCR protocols. A guide to methods and applications. Academic Press Inc., Harcourt Brace Jovanovich, New York.

16. Jean, B.R., M. Madritch, C.L. Trout, and G. Parra. 1998. PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. *Appl. Environ. Microbiol.* 64, 948-954.

17. Kim, K.S., H.S. Shin, H.J. Kim, S.J. Woo, Y.I. Ham, K.Y. Shin, J.W. Lee, B.S. Kim, S.O. Shim, M.W. Lee, and Y. S. Lee. 1999. Molecular genetic classification of *Phytophthora* species and *P. infestans*-specific marker selection by RAPD finger-printing. *Korean J. Mycology* 27(6), 394-398.

18. Kim, K.S. and Y. S. Lee. 2000. Rapid and accurate species-specific detection of *Phytophthora infestans* through analysis of ITSII region in rDNA. *J. Microbiology and Biotechnology.* 10, 651-655.

19. Lee, S.B. and J.W. Taylor. 1992. Phylogeny of true fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Mol. Biol. Evol.* 9, 636-653.

20. Lee, Y.S., H.S. Choi, and K.S. Kim. 1998. Analyses of genetic relationships of *Rhizoctonia solani* isolated from various crop species and rapid identification of anastomosis group with RAPD method. *Korean J. Mycology* 26, 373-379.

21. McPherson, M.J., R.J. Oliver, and S.J. Gurr. 1992. The polymerase chain reaction. p. 123-145. *In* Gurr S.J., M.J. McPherson, and D.J. Bowles (eds.), Molecular plant pathology, vol. 1. A practical approach. IRL Press, New York.

22. Mesguita, A.G.G., T.J. Paula Jr., M.A. Moreira, and E.G. de Barros. 1998. Identification of races of *Colletorichum lindemuthianum* with the aid of PCR-based molecular markers. *Plant Disease* 82, 1084-1087.

23. Nazar, R.N., X. Hu, J. Schmidt, D. Culham, and J. Robb. 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* wilt pathogens. *Physiol. Mol. Plant Pathol.* 39, 1-11.

24. Old, R.W. and S.B. Primrose. 1994. Polymerase chain reaction. p. 178-190. *In* Principles of Gene Manipulation. Blackwell Science Inc., Cambridge, MA. USA.

25. O'Donnell, K. 1992. Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum (Gibberella Pulicaris).* *Curr. Genet.* 22, 213-220.

26. Paabo, S. 1990. Amplifying ancient DNA. p. 159-166. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White (eds.), PCR protocols. A guide to methods and applications. Academic Press Inc., Harcourt Brace Jovanovich, New York.

27. Panabieres, F., A. Marais, F. Trentin, P. Bonnet, and P. Ricci. 1989. Repetitive DNA polymorphism analysis as a tool for identifying *Phytophthora* species. *Phytopathology* 79, 1105-1109.

28. Paran, I. and R.W. Michelmore. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet.* 85, 985-993.

29. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

30. Shattock, R.C., P.W. Tooley, and W.E. Fry. 1986. Genetics of *Phytophthora infestans:* Determination of recombination, segregation, and selfing by isozyme analysis. *Phytopathology* 76, 410-413.

31. Spielman, L.J. 1991. Isozymes and the population genetics of *Phytophthora infestans.* p. 231-241. *In* J.A. Lucas, R.C. Shattock, D.S. Shaw, and L.R. Cooke (eds.), Phytophthora. Cambridge Univ. Press, Cambridge, U.K.

32. Tooley, P.W. 1988. Use of uncontrolled freezing for liquid nitrogen storage of *Phytophthora* species. *Plant Disease* 72, 680-682.

33. Tooley, P.W., W.E. Fry, and M.J. Villareal-Gonzalez. 1985. Isozyme characterization of sexual and asexual *Phytophthora infestans* populations. *J. Hered.* 76, 431-435.

34. Tooley, P.W., B.A. Bunyard, M.M. Carris, and E. Hatziloukas. 1997. Development of PCR primers from internal transcribed spacer region 2 for detection of *Phytophthora* species infecting potatoes. *Appl. Environ. Microbiol.* 63, 1467-1475.

35. Trout, C.L., J.B. Ristaino, M. Madritch, and T. Wangsoomboondee. 1997. Rapid detection of *Phytophthora infestans* in late blight infected potatoes and tomatoes using PCR. *Plant Disease* 81, 1042-1048.

36. Vakalounakis, D.J. and G.A. Fragkiakakis. 1999. Genetic diversity of *Fusarium oxysporum* isolates from cucumber: Differentiation by pathogenicity, vegetative compatibility, and RAPD fingerprinting. *Phytopathology* 89, 161-168.

37. Wang, H., M. Qi, and A.J. Cutler. 1993. A simple method of preparing plant samples for PCR. *Nucleic Acids Res.* 21, 4153-4154.

38. White, T.J., T. Burns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. p. 315-322, *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.), PCR Protocols. Academic Press, San Diego, CA, USA..

39. Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18. 6531-6535.

40. Yao, C., R.A. Frederiksen, and C. Macgill. 1992. Length heterogeneity in ITS2 and the methylation status of CCGG and GCGC sites in the rRNA genes of the genus *Peronosclerospora.* *Curr. Genet.* 22, 415-420.