Specific Detection of *Pythium aphanidermatum* from Hydroponic Nutrient Solution by Booster PCR with DNA Primers Developed from Mitochondrial DNA

P.H. Wang,[1,*] L.M. Boo,[1] Y.S. Lin[2] and Y. Yeh[3]

Pythium aphanidermatum causes damping-off and root rot of vegetable crops in hydroponic systems. A DNA probe was isolated and modified from a library of *Hin*dIII-digested mitochondrial DNA of *P. aphanidermatum* that strongly hybridized to DNA of *P. aphanidermatum* and weakly hybridized to DNA of *Pythium deliense*. Cross-hybridizing sequences were absent from DNA of plants and other related fungi. The probe detected as little as 5 ng of *P. aphanidermatum* DNA and 250 ng of *P. deliense* DNA in slot-blot assays. *P. aphanidermatum* was detected by a hybridization assay of total DNA extracted directly from infected roots. A pair of oligonucleotide primers P1 and RP2, which allowed amplification of a specific 0.65 kb DNA fragment of *P. aphanidermatum* using polymerase chain reaction (PCR), was designed from a specific DNA probe. Specific amplification of this fragment from *P. aphanidermatum* was highly sensitive, detecting template DNA as low as 0.1 pg total DNA by booster PCR. Specific booster PCR amplification using P1 and RP2 was successful in detecting *P. aphanidermatum* in naturally infected nutrient solution and roots of vegetables in a field hydroponic system.

KEY WORDS: *Pythium aphanidermatum*; mitochondrial DNA; booster PCR; hydroponics; detection.

INTRODUCTION

Glasshouse hydroponic systems that utilize a reservoir for recirculating nutrient solution to and from growth chambers are common in commercial vegetable production (9). If a root pathogen is accidentally introduced into such a system at any location, rapid and uniform distribution may occur. In summer, *Pythium aphanidermatum* (Edson) Fitzp. has been identified as the most common and destructive root-infecting pathogen of vegetable crops in hydroponic systems in Taiwan (7). Plant cultivars can differ widely in levels of resistance to *P. aphanidermatum*. Not all species of *Pythium* are pathogenic and pathogenicity can vary between species (11,13). Therefore, reliable methods for identification of *P. aphanidermatum* are important requirements for crop protection in hydroponic systems.

There is a need to develop a forecasting system and/or cultural or chemical protection strategies, which would reduce inoculum level below the minimum necessary for host

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colonization and disease development (28). Current methods are based on (*a*) baiting, (*b*) isolation of the pathogen on selective media, or (*c*) microscopic methods (8,24,27). However, various problems exist in the practical application of a baiting method in the field that cannot be solved (26,29). Culturing is time-consuming and not very sensitive, while microscopic detection lacks sensitivity and specificity. Molecular genetics offers a promising alternative for rapid identification and detection of *Pythium* species which could help the hydroponic industry prevent major losses.

Species-specific molecular probes are a powerful means for detecting *Pythium* in soil and plant samples. DNA probes for several species of *Pythium* have been reported. Martin (15) derived probes for *P. oligandrum* and *P. sylvaticum* from restriction fragments of mtDNA. Lévesque *et al.* (12) used part of the internal transcribed spacer of the ribosomal DNA to synthesize oligonucleotide probes that can be used to identify *P. aphanidermatum, P. ultimum, P. acanthicum* and *Phytophthora cinnamomi.* Matthew *et al.* (19) found a repeated sequence in a genomic library of *P. irregulare* that recognized *P. irregulare* and *P. spinosum* isolates. Klassen *et al.* (10) amplified the 5S rRNA spacer regions from *P. intermedium, P. macrosporum, P. sylvaticum, P. ultimum, P. okanoganense, P. anandrum, P. acanthicum* and *P. mastophorum* and used them as species-specific probes.

In fungi, the mitochondrial DNA (mtDNA) is small and present in high copy number (31), which makes it suitable for restriction enzyme analysis and provides a powerful tool in taxonomy. When comparisons were made using mtDNA, the variation within species in genera such as *Phytophthora* (4), *Aspergillus* (22), *Colletotrichum* (27) and *Trichoderma* (23) was high. In contrast to these observations, restriction endonuclease-digested mtDNA from 29 *Pythium* species showed distinctly different species-specific banding patterns (14,17). One possible reason for this is that the mitochondrial genome in the genus *Pythium* had a high level of conservation of restriction sites within a species compared with the low level among species. The mitochondrial genome in the genus *Pythium* is a circular molecule and contains the inverted repeat (IR) structure (20,21). The IR structures in *Pythium* may comprise ~80% of the genome (20). Restriction endonuclease-digested mtDNA-banding patterns can be a useful character for species identification in the genus *Pythium.*

In order to find effective methods for detecting *P. aphanidermatum*, mtDNA restriction fragment length polymorphism (RFLP) patterns of 15 *Pythium* species were compared and the specific fragments of mtDNA were selected as probes. The specificity of digoxigenin (DIG)-labeled probes was determined by slot-blot hybridization assays for the detection of *P. aphanidermatum* and *P. deliense*. To improve the specificity of the method, the mtDNA probe was recognized as a potential site for the selection of oligonucleotide primers for diagnostic polymerase chain reaction (PCR) assays. This paper describes the selection of a species-specific PCR primer pair constructed from a cloned mtDNA fragment and its use for the detection of *P. aphanidermatum* from roots and infected hydroponic nutrient solution.

MATERIALS AND METHODS

Fungal isolates and plant materials Isolates of *Pythium* species used in this study are listed in Table 1. Cultures were maintained at room temperature on potato dextrose agar (PDA, Difco, USA) slants overlaid with mineral oil (Sigma Chemical Co., St. Louis, IL, USA). An active culture of each isolate was obtained by culture in PDA and incubation at

28°C. After 3 days, two 0.5-cm-diam actively growing hyphal regions from agar blocks were cut and placed into 50 ml of V-8 broth (200 ml V-8 juice; Campbell Co., Toronto, Canada), 2.5 g $CaCO_3$, 800 ml distilled water; this was centrifuged at 8,000 rpm for 30 min, the supernatant autoclaved, placed in a 500-ml flask and incubated for 3 days at 28°C. The culture was filtered through four layers of cheesecloth and washed with sterile distilled water. The mycelial mat was stored at -70°C until DNA was extracted.

Seeds of the plant species *Amaranthus mangostanus* L., *Brassica chinensis* L., *Cucumis melo* L., *Cucumis sativus* L., *Ipomoea reptans* (L.) Poir. and *Pisum sativum* L. were obtained from commercial sources. Seeds were surface sterilized during 1 min in 1% sodium hypochlorite and then washed twice in sterile distilled water. Seedlings were grown on 2% water agar in petri dishes at room temperature.

DNA extraction Total genomic DNA of fungal isolates was extracted according to the procedure described by Ausubel *et al.* (1); mitochondrial DNA was collected by CsCl-bisbenzimide density gradient centrifugation as described by Garber and Yoder (5), and stored at -20°C; genomic DNA was extracted from plant seedlings by the CTAB method described by Wang and White (32) as adapted from Doyle and Doyle (3).

Restriction enzyme digestions In order to assess sequence variability, mtDNA was used for restriction enzyme analysis. Ten μg of mtDNA was digested for 16 h at 37°C with 30 units of *Hae*III, *Hin*dIII, *Msp*I, *Pst*I, or *Rsa*I (Roche, Mannheim, Germany). The digested DNA fragments were separated on a 0.8% agarose (Promega Corp., Madison, WI, USA) gel with Tris-acetic acid-EDTA (TAE) buffer. Phage λ DNA digested with *Hin*dIII was used as the size standard.

Cloning of mtDNA and screening of recombinants Approximately 20 μg of CsCl-purified mtDNA of *P. aphanidermatum* was digested with *Hin*dIII at 37°C for 12 h. Resulting fragments were ligated with dephosphorylated *Hin*dIII-digested pUC19, using a 3:1 insert/vector ratio, at 16°C for 6 h. Recombinant DNA was used to transform competent cells of *Escherichia coli* JM105. White colony transformants that grew on a Luria-Bertani medium (25) containing X-gal (5-bromo-4-chloro-3-indoyl-ß-galactoside, 0.5 mM), IPTG (isopropyl-ß-D-thiogalacto-pyranoside, 40 g ml^{-1}), and carbenicillin (40 g ml^{-1}) were subcultured. Plasmid DNA was extracted from white colonies by the alkaline lysis method (25).

Probe purification and labeling Restriction fragments were generated by digestion of the plasmid DNA with *Hin*dIII. Fragments were separated in 0.8% agarose gels and purified with the Wizard PCR preps DNA purification system (Promega). Purified DNA fragments were labeled with Digoxigenin-11-dUTP using the random primed method (Roche). Labeled probes were stored at -20°C.

Hybridization procedures DNA samples of 1 μg were denatured by adding 100 μl denaturation solution (0.5 M NaOH, 1.5 M NaCl), and then treated with 100 μl neutralization solution (1.0 M Tris-HCl, pH 7.0; 1.5 M NaCl). Denatured DNA samples were blotted onto a nitrocellulose membrane with the Bio-Slot microfiltration (Hoeffer PR600, Amersham Biosciences, San Francisco, CA, USA) and bound to the membrane by UV-crosslinking. Membrane strips were prehybridized at 68°C for 2–3 h in a sealed plastic bag in 5 × SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, and 1% (w/v) blocking reagent. After prehybridization, the labeled probe was added to the hybridization solution,

the bag was resealed, and hybridization was done at 68°C in 10-ml volumes for 12–16 h; the manufacturer's procedure was used for high-stringency washes. Digoxigenin was detected by chemiluminescence, using alkaline phosphatase–anti-digoxigenin antibody.

TABLE 1. *Pythium* isolates used in this study

Species	Isolate*	Source	Host/Habitat[v]
P. acanthicum	HRI/S167[x]	UK	*Malus pumila*
P. aphanidermatum	HRI/PA1[xw]	UK	*Daucus carota*
P. aphanidermatum	YSL/P2[zyw]	USA	–
P. aphanidermatum	YSL/P42[w]	Taiwan	*Cucumis melo*
P. aphanidermatum	YSL/P50[w]	Taiwan	*Momordica charantia*
P. aphanidermatum	YSL/P57[w]	Taiwan	*Pisum sativum*
P. aphanidermatum	AVRDC/Pyth-2[w]	Taiwan	*Brassica oleracea* var. *capitata*
P. aphanidermatum	AVRDC/Pyth-18[w]	Taiwan	*Lycopersicon esculentum*
P. aphanidermatum	AVRDC/Pyth-26[w]	Taiwan	*Capsicum annuum*
P. aphanidermatum	CWH/104[w]	Taiwan	*Cucumis sativus*
P. aphanidermatum	CWH/701[w]	Taiwan	*Brassica chinensis*
P. aphanidermatum	CWH/702[w]	Taiwan	*Citrullus vulgaris*
P. aphanidermatum	CWH/705[w]	Taiwan	*Amaranthus mangostanus*
P. aphanidermatum	CWH/707[w]	Taiwan	*Luffa cylindrica*
P. arrhenomanes	HRI/P. arr[x]	UK	*Daucus carota*
P. aristosporum	YSL/P37[zyx]	Taiwan	*Cucumis melo*
P. catenulatum	HRI/JW[x]	UK	*Rorippa nasturtium-aquaticum*
P. catenulatum	YSL/P23[y]	USA	–
P. coloratum	IMI/181938[x]	New Zealand	*Daucus carota*
P. coloratum	YSL/P3[y]	Taiwan	–
P. deliense	IMI/342678[x]	France	*Persea americana*
P. deliense	YSL/P4[zy]	USA	–
P. deliense	AVRDC/Pyth-24	Taiwan	*Capsicum annuum*
P. dimorphum	YSL/P33[yx]	Taiwan	*Euphorbia longana*
P. dissotocum	YSL/P341[yz]	Taiwan	*Lactuca sativa*
P. dissotocum	YSL/P22[y]	USA	–
P. graminicola	HRI/P. gram[x]	UK	–
P. graminicola	YSL/P27[zy]	Taiwan	–
P. heterothallicum	HRI/S4[x]	UK	*Malus pumila*
P. hydnosporum	YSL/P294[x]	Taiwan	*Brassica* sp.
P. inflatum	IMI/308148[z]	–	–
P. intermedium	HRI/COM3[x]	UK	*Daucus carota*
P. irregulare	YSL/P263[yx]	Taiwan	*Allium fistulosum*
P. macrosporum	YSL/P24[y]	Taiwan	–
P. mamillatum	HRI/P. mam[x]	UK	*Daucus carota*
P. mamillatum	YSL/P8[zy]	USA	–
P. mamillatum	YSL/P38[y]	Taiwan	*Cucumis melo*
P. myriotylum	YSL/P63[zy]	Taiwan	*Brassica chinensis*
P. myriotylum	YSL/P118[y]	Taiwan	–
P. myriotylum	YSL/P130[x]	Taiwan	*Brassica chinensis*
P. nunn	IMI/324024[z]	USA	Soil
P. oligandrum	HRI/P. olig[x]	UK	*Daucus carota*
P. oligandrum	YSL/P9[zy]	USA	–
P. paroecandrum	HRI/P. paro[x]	UK	*Rorippa nasturtium-aquaticum*
P. paroecandrum	YSL/P10[zy]	USA	–
P. periilum	YSL/P43[zy]	USA	*Brassica chinensis*

Table 1. (Cont'd.)

Species	Isolate*	Source	Host/Habitat[v]
P. polymastum	HRI/P. poly[x]	UK	*Brassica oleracea*
P. rostratum	HRI/P. ros[x]	UK	*Daucus carota*
P. salpingophorum	HRI/L50[x]	UK	*Lactuca sativa*
P. spinosum	HRI/P. spino[x]	UK	*Daucus carota*
P. spinosum	YSL/P30[zy]	Taiwan	–
P. splendens	HRI/S138[x]	UK	*Malus pumila*
P. splendens	YSL/P25[zy]	USA	–
P. sulcatum	HRI/LMW1[x]	UK	*Daucus carota*
P. sylvaticum	HRI/OI[x]	UK	*Allium cepa*
P. sylvaticum	YSL/P13[zy]	USA	–
P. torulosum	YSL/P16[zyx]	USA	–
P. tracheiphilum	HRI/2A[x]	Spain	*Lactuca sativa*
P. ultimum var. *ultimum*	HRI/PU[x]	UK	Soil
P. ultimum var. *ultimum*	YSL/P18[zy]	USA	–
P.u. var. *sporangiiferum*	IMI/308275[x]	UK	Soil
P. vanterpoolii	HRI/S172[x]	UK	-
P. vexans	IMI/132189[x]	Netherlands	*Lycopersicon esculentum*
P. violae	HRI/IPV1[x]	Israel	*Daucus carota*
P. volutum	IMI/331766[x]	Japan	*Triticum* sp. and *Hordeum* sp.
Pythium Group G	YSL/P90[zy]	Taiwan	*Brassica chinensis*
Pythium Group F	YSL/P211[zy]	Taiwan	*Hibiscus* sp.
Phytophthora cryptogea	HRI/P. cry	UK	*Fragaria chiloensis*

* AVRDC, Asian Vegetable Research and Development Center, Taiwan; CWH, collections of Prof. C.W. Huang, Department of Plant Pathology, National Chung-Hsing University, Taiwan; HRI, Horticulture Research International, Wellesbourne, Warwick, UK; IMI, International Mycological Institute, Surrey, UK; YSL, collections of Prof. Y.S. Lin, Department of Plant Pathology, National Chung-Hsing University, Taiwan. The *Pythium* isolates from the USA were provided by Prof. M.E. Stanghellini, University of California, Riverside, CA, USA.

[z] *Pythium* isolates used in Fig. 1 (mtDNA RFLP).

[y] *Pythium* isolates used in Fig. 2 (slot-blot hybridization).

[x] *Pythium* isolates used in Fig. 4 (specific test).

[w] *Pythium aphanidermatum* isolates used in Fig. 7.

[v] – not known.

Detection of *P. aphanidermatum* in pea roots by slot-blot hybridization A filter paper (Whatman) was moistened with water in a 9-cm petri dish and autoclaved for 1 h. Peas were seeded (six seeds per dish) aseptically and kept at 25°C. After 7–10 days, when the seedlings were at the cotyledon stage and with roots approximately 3–4 cm long, they were thinned to four seedlings per dish. Four colonized 0.5 cm^2 agar disks taken from a 2-day-old culture of *P. aphanidermatum* on PDA were placed near the root tips. Roots of uninfested plates served as controls. Symptoms developing on the seedling were observed. These included damping-off, root rot and watery rot of stems. After 7 days, plants were removed; the pathogen was re-isolated and identified. DNA was extracted from roots. Probe pJM73-2 was used to detect *P. aphanidermatum* from artificially infected pea roots. The experiment was repeated twice. Another clone was selected from the same library, that containing a plasmid pJM70.

Sequencing of the probe and primer design Probe pJM70 was sequenced from plasmid pJM70 with the pUC19 forward and reverse sequencing primers according to the manufacturer's instructions of CircumVent^{TM} Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs, Beverly, MA, USA). Five 20 or 21-mers' oligonucleotide primers were selected from the partial sequence of specific DNA probe pJM70.

Specificity and sensitivity of PCR amplification The six PCR primer pair combinations of five primers were tested using PCR amplifications on total DNA of 34 *Pythium* and one *Phytophthora* species (Table 1). PCR was performed in a 25 μl PCR mixture which contained 12.5 ng DNA, 100 nM of each primer, 1.0 unit *Taq* DNA polymerase, 50 μM of each dNTP in a PCR reaction buffer. The mixture was overlaid with 50 μl mineral oil. The PCR mixtures were amplified at a higher stringency, DNA denaturation at 94°C, 2 min for the first cycle and 1 min for subsequent 40 cycles, 20 sec at 68°C for primer annealing, 5 sec at 72°C for extension, and a final cycle of 1 min at 72°C. PCR amplifications containing no DNA template were used as controls in every experiment to test for the presence of contamination of reagents and reaction mixtures. According to the preliminary test, PCR with 40 cycles was not sensitive enough for field detection. The sensitivity of booster PCR was tested as follows. A serial dilution of *P. aphanidermatum* total DNA was used to determine sensitivity of the PCR amplification. The optimal concentration of primers, the thermal profile, extension time, amount of input DNA, and other parameters were examined in preliminary experiments to establish the reproducibility of the PCR amplification, maximize its yield and stringency, minimize the reaction time and ensure amplification specificity. A 50 μl PCR mixture contained 25 ng DNA, 100 nM of each primer (P1/RP2), 1.0 unit *Taq* DNA polymerase, 20 μM of each dNTP in a PCR reaction buffer. The mixture was overlaid with 50 μl mineral oil. The amplification protocol consisted of one single denaturation at 94°C for 2 min, 20 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 20 sec, extension at 72°C for 5 sec, and a final extension at 72°C for 2 min. All experiments were conducted in a thermalcycler (OmniGene, Hybaid, Middlesex, UK), which worked under tube control. For the second PCR stage, 5 μl of the first PCR product was used as a template in a 50 μl PCR mixture. The mixture contained 100 nM of each primer, 2.0 units *Taq* DNA polymerase, and 50 μM of each dNTP. Subsequent amplification reactions were performed with 50 cycles.

Detection of *P. aphanidermatum* in field samples PCR-based detection of *P. aphanidermatum* was tested using DNA extracted from naturally infected nutrient solutions and infected roots of *Amaranthus mangostanus* L. and *Brassica chinensis* L. collected from Youth Hydroponic Farm, Taichung, Taiwan. DNA extraction from plant tissues was described above. The nutrient solution was collected from the center of the trough and 50 ml was passed through a Nucleopore filter (#140413, Nucleopore Corp., Pleasanton, CA, USA). The filters were transferred into microtubes and DNA was extracted by the CTAB-mini DNA extraction method as described previously.

RESULTS

Mitochondrial DNA RFLPs of 15 *Pythium* species were assessed using *Hae*III, *Hin*dIII, *Msp*I, *Pst*I and *Rsa*I. *Hin*dIII proved to be the most informative for separating these species (Fig. 1).

In order to construct a mtDNA library, CsCl-purified mtDNA of *P. aphanidermatum* was digested with *Hin*dIII and ligated into *Hin*dIII-digested plasmid pUC19. One plasmid, pJM73, containing a 2.4 kb insert of *P. aphanidermatum* mtDNA fragment, was selected for evaluation as a species-specific probe from 40 clones. Twenty-five of the *Pythium* spp. listed in Table 1 were examined by slot-blot hybridization. When the pJM73 was used as a probe, it hybridized to total DNA extracts from isolates of *P. aphanidermatum*, *P. deliense*, *P. oligandrum*, *P. torulosum*, *P. aristosporum*, *P. spinosum*, *P. myriotylum* and *P. periilum*.

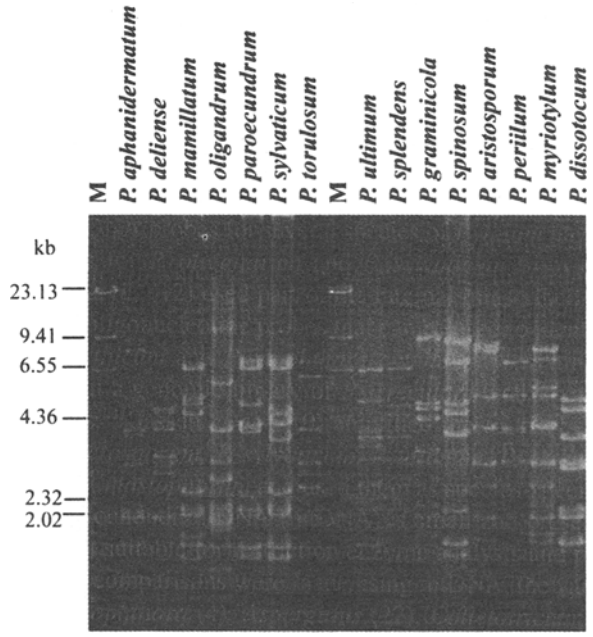

Fig. 1. Agarose gel electrophoresis of *Hin*dIII-digested mitochondria DNA (mtDNA) restriction fragment length polymorphism (RFLP) patterns of 15 *Pythium* species. DNA digested with *Hin*dIII as the size marker (M).

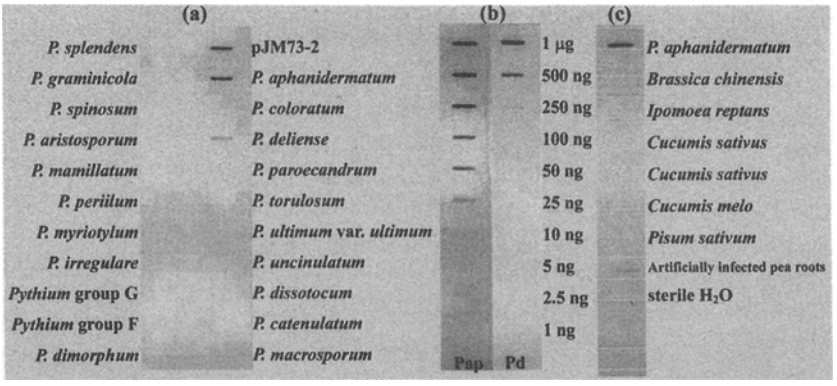

Fig. 2. Slot-blot hybridization of probe pJM73-2 to *(a)* total DNA of 21 *Pythium* species and pJM73-2, *(b)* serial dilution of genomic DNA of *P. aphanidermatum* (Pap) and *P. deliense* (Pd), and *(c)* DNA of six plant species and pea roots artificially infected by *P. aphanidermatum*.

In order to improve the specificity, pJM73 was digested with *Msp*I and a 1.55-kb fragment, pJM73-2, was obtained.

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AF302790                          Primer P1                Primer P3                Primer P5
pJM70    1  GGTTTTCGAC GGTAGTTCGT TTGTTTGGGG ATACGTTACG AGTATTTAGC GGGTCGGAGG AGGTTTGGGC GATGCGTGAG TNGTCGGAAC GNNGTTTTTT
          101  GGTGTTTTGC GAGTTTGTTT TTTCGTGCAA AAAAGTGAAT AGCGGTGCGA GTTGGAAAGC TTTTTCGTTT CTGCAAAGTG TGGAAGGTGC GCTGTAGCAT
          201  TTGTCGAGCG TGTGCAAGTT TTACTGCGCG GTGAGTGCTG TGTCA

AF302791
pJM70    1  GCTGTTTCGG TCAGATTGAG GTAAGTTTCG TGGAAGCCAG TAGTGCCAAA TATCGAAGCG GGATTCGATG CGCATACCTA CGGGGCATAT GTGGACGAAC
          101  TTTGGCGGTC GCAGGGTCGG TGGTTGTGTC GGGATGGCGG CGCGCGTGGC GGCGGAGGCG CTGTTTGGGA GGTGTCGAGT GGTGTCGCTG GTTGGTTAAG
          201  TACGAAGTTT GTGGTAAATT ACGGCATCGC AGTTTTTTAT GCGGATTGCT TCCC                                    Primer RP2
                          Primer RP4
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Fig. 3. Partial sequence of the insert from pJM70, GenBank accession numbers AF302790 and AF302791. The sequences corresponding to the primers P1, P3, P5, RP2 and RP4 are underlined.

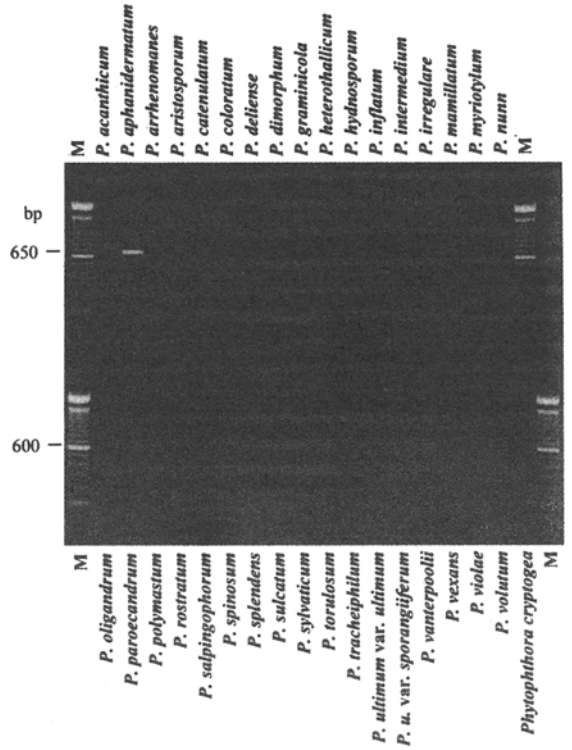

Fig. 4. Polymerase chain reaction (PCR) amplification of 34 *Pythium* and one *Phytophthora* species. DNA was analyzed by 1.2% agarose gel electrophoresis. Lanes M, 100 bp DNA ladder marker.

Specificity of pJM73-2 was evaluated by hybridization to total DNA of 21 *Pythium* spp., 18 other fungal species, and six plants. When probed with pJM73-2, a high degree of species specificity to *P. aphanidermatum* and a weak reaction to *P. deliense* were achieved when tested by slot-blot hybridization against 20 other *Pythium* species (Fig. 2a). Hybridization of serial dilutions of 1 μg to 1 ng genomic DNA of *P. aphanidermatum* and *P. deliense* indicated that ~5 ng of *P. aphanidermatum* and 250 ng of *P. deliense* DNA could be detected (Fig. 2b). Hybridization was also not detected with DNA extracted from

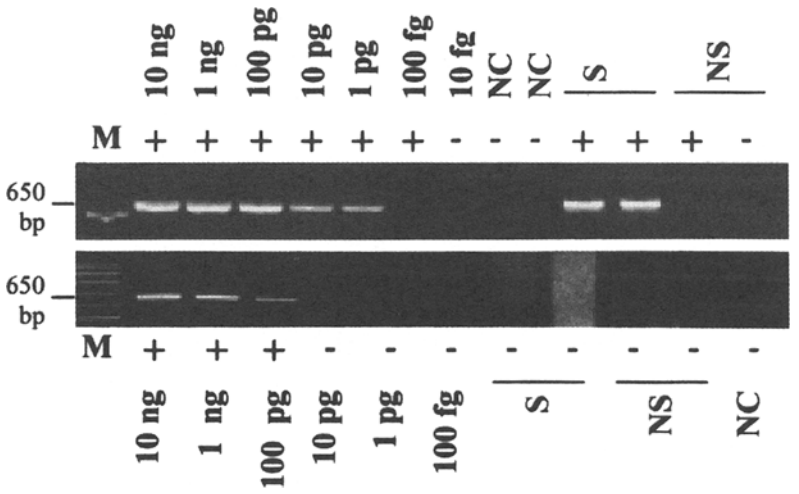

Fig. 5. Gel electrophoresis of booster PCR (upper) and PCR (lower) amplified specific product (650 bp) from tenfold serial dilutions of *Pythium aphanidermatum* DNA and DNA extracted from roots of hydroponically grown *Amaranthus mangostanus* (amaranth) with (S) or without (NS) symptoms. Lanes M, 100 bp DNA marker; lanes NC, negative control reaction without template DNA.

host plants such as *Brassica chinensis*, *Ipomoea reptans*, *Cucumis sativus*, *Cucumis melo* and *Pisum sativum* (Fig. 2c). Hybridization of DIG-labeled pJM73-2 probe enabled the detection of *P. aphanidermatum* from artificially infected pea roots (Fig. 2c). When probed with a smaller insert (0.7 kb) of clone pJM70 from *P. aphanidermatum* strain P2, similar reactions as with probe pJM73-2 were observed (data not shown).

A species-specific primer pair was designed from a DNA fragment of pJM70 to simplify the detection procedure. An insert of pJM70 was partially sequenced with primers from plasmid pUC19. A 245 bp forward sequence (GenBank accession no. AF302790) and a 254 bp reverse sequence (GenBank accession no. AF302791) were obtained (Fig. 3). Primers P1 (5'- GTT CGT TTG TTT GGG GAT ACG), P3 (5'- TTA CGA GTA TTT AGC GGG TCG), P5 (5'- GGA GGT TTG GGC GAT GCG TG), RP2 (5'- CTT CGT ACT TAA CCA ACC AGC) and RP4 (5'– GCG ATG CCG GTA ATT TAC CAC) were constructed from the sequence of probe pJM70 (Fig. 3). Specificity was tested with different primer combinations. Under optimal conditions, primer pair P1/RP2 specifically amplified a 650 bp fragment of the mtDNA from isolates of *P. aphanidermatum* and did not amplify DNA from the other *Pythium* species listed in Table 1 (Fig. 4). Primer pair P1/RP4 amplified DNA of some *Pythium* spp. and plants. Primer pairs P3/RP4 and P3/RP2 amplified a single band from many *Pythium* spp., whereas P5/RP2 and P5/RP4 amplified several bands from many *Pythium* spp. (data not shown). The primer pair P1/RP2 was found to be the most specific of the combinations tested for the specific detection of *P. aphanidermatum*.

Amplifications of serial dilutions of 10 ng to 100 fg total DNA of *P. aphanidermatum* indicated that approximately 100 pg of *P. aphanidermatum* DNA could be detected by standard PCR (40 cycles) using primer pair P1/RP2. Booster PCR (20+50 cycles) detected 0.1 pg of *P. aphanidermatum* DNA (Fig. 5). The ability of P1/RP2 to detect *P. aphanidermatum* in diseased plants was tested by isolating total DNA from field samples.

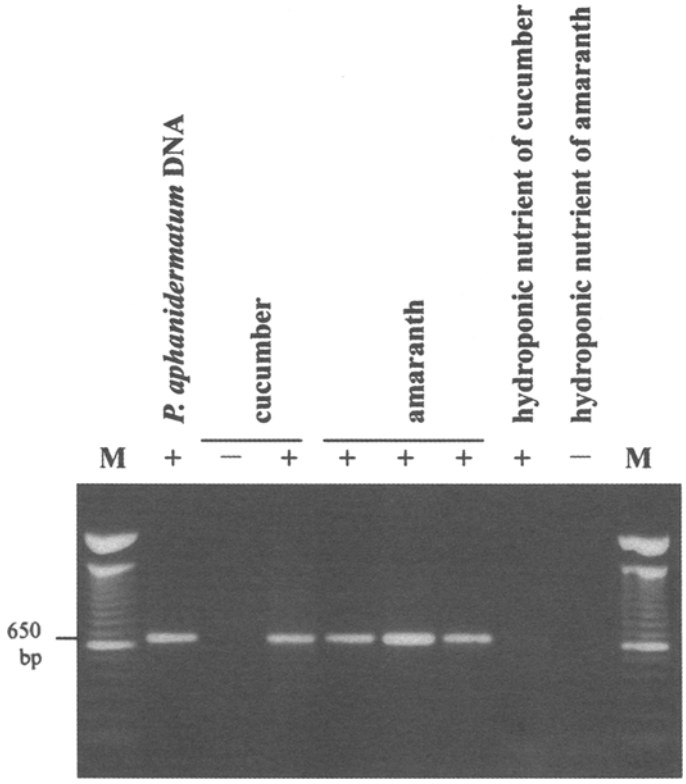

Fig. 6. Detection of *Pythium aphanidermatum* in the hydroponic nutrient and roots of cucumber and amaranth. Gel electrophoresis of booster PCR, amplifying the specific product (650 bp) from DNA extracted from the roots of hydroponically grown cucumber and amaranth. Lanes M, 100 bp DNA marker; lanes –, negative control reaction without template DNA.

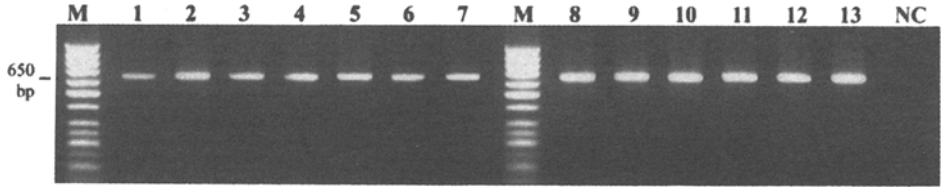

Fig. 7. Gel electrophoresis of 13 *Pythium aphanidermatum* isolates listed in Table 1, amplified by using standard PCR. Lanes M, 50 bp DNA marker; lane NC, negative control reaction without template DNA.

The specific product of *P. aphanidermatum* was amplified from two root samples of *A. mangostanus* (amaranth) collected from a hydroponic farm by booster PCR but not by standard PCR. Booster PCR detected the pathogen from two samples with observed root rot symptoms and a root sample without symptoms (Fig. 5). Furthermore, the pathogen was detected from the roots of cucumber and amaranth with symptoms of damping off or

root rot, by booster PCR. The product was obtained from hydroponically grown cucumber but not the nutrient-grown amaranth (Fig. 6), and also from 13 other *P. aphanidermatum* isolates from a worldwide collection (Fig. 7).

DISCUSSION

The use of DNA probes for diagnostics offers advantages in specificity and sensitivity. In this study, a DNA probe (pJM73-2) was isolated and modified from a library of *Hin*dIII-digested mitochondrial DNA that strongly hybridized to DNA of *P. aphanidermatum* and weakly hybridized to DNA of *P. deliense* in slot-blot assays. Of the specificity tests, the slot-blot analysis revealed that *P. aphanidermatum* and *P. deliense* were identified among 21 *Pythium* species, and *P. aphanidermatum* was detected by a hybridization assay of total DNA extracted directly from infected roots.

Pythium aphanidermatum and *P. deliense* are morphologically similar, with smooth oogonia, aplerotic oospores and inflated filamentous sporangia. These two species are differentiated mainly by the curvature of the oogonial stalk in *P. deliense* (24). Both of them are typical plant parasites of warm regions and widely distributed in Taiwan. Based on the sequence analysis of the mitochondrially encoded cytochrome oxidase II gene (16), RAPD (6), RFLP (32) and sequences analysis (18) of the internal transcribed spacers (ITS) regions, *P. aphanidermatum* and *P. deliense* are closely related species.

Recent developments in gene technology have enabled the synthesis of different genetic probes that permit rapid identification of *Pythium* species. Lévesque *et al.* (12) used regions of the ITS of ribosomal DNA to design probes specific for *Pythium* species. The 29-mer probe developed for *P. aphanidermatum* cross-hybridized with some other *Pythium* species at 55°C, but when the assay temperature was increased to 60°C, cross-hybridization with only *P. deliense* was observed (12). It is difficult to distinguish *P. aphanidermatum* and *P. deliense* by the probe designed from the ITS region, since there are only a few base differences within the ITS region differentiating between the two species.

To improve the specificity of the method, a mtDNA probe was considered as a potential site for the selection of oligonucleotide primers for diagnostic PCR assays. The primer pair P1 and RP2 was found to be the most specific of the combinations tested for the specific detection of *P. aphanidermatum*. The forward primers P3 and P5, and reverse primers RP4 showed homology to the sequence of some other *Pythium* species. However, the primer pair P1 and RP2 did not amplify DNA from *P. deliense* nor any other tested *Pythium* species. The specific PCR of product of 650 bp was detected in all 13 isolates of *P. aphanidermatum* collected from different hosts and various geographical regions (Fig. 7). It is expected that the PCR-based assay developed in this study should detect all *P. aphanidermatum* isolates regardless of origin. No PCR product was obtained from three isolates of *P. deliense*, and we demonstrated that with P1 and RP2, the PCR method clearly distinguished between *P. aphanidermatum* and *P. deliense*.

In our study, standard PCR was more sensitive than Southern blot hybridization. However, due to the sensitivity limitation of PCR, some field samples failed to produce an amplification product by standard PCR. The detection limit was decreased to 0.1 pg total DNA by booster PCR. Booster PCR enhanced the sensitivity and prevented the false-positive reactions of field samples with a low concentration of target fungal DNA. In the field survey, the technique detected *P. aphanidermatum* in total preparations from naturally infected plant roots or hydroponic nutrient solution. The booster PCR technique

enabled multiple individual samples to be detected simultaneously, and the entire test to be completed within 8 h.

Devastating crop losses caused by *P. aphanidermatum* have occasionally been reported (2,9,30), and because effective control measures are lacking, cultivation of susceptible crops has sometimes been abandoned (2). This is the limiting factor of growing *Brassica chinensis* and *Lactuca sativa* in Taiwanese commercial hydroponic farms in the summer. The low concentration of zoospores or cysts in nutrient solution makes the use of PCR desirable in DNA-based detection methods. *P. aphanidermatum* could be detected directly by amplifying the DNA with species-specific primers without culturing or baiting. This technique may be used to investigate the population fluctuation of the pathogen in hydroponic systems and may help the farmer develop disease management strategies.

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