Detection of *Pythium ultimum* Using Polymerase Chain Reaction with Species-Specific Primers

K. Kageyama, Faculty of Agriculture, Gifu University, Gifu 501-11, Japan; A. Ohya, National Research Institute of Vegetables, Ornamental Plants and Tea, Ano, Mie 514-23, Japan; and M. Hyakumachi, Faculty of Agriculture, Gifu University, Gifu 501-11, Japan

ABSTRACT

Kageyama, K., Ohya, A., and Hyakumachi, M. 1997. Detection of *Pythium ultimum* using polymerase chain reaction with species-specific primers. Plant Dis. 81:1155-1160.

This study was conducted to sequence the rDNA internal transcribed spacer (ITS) region of *Pythium ultimum* and *Pythium* group HS, design species-specific primers for polymerase chain reaction (PCR), and detect *P. ultimum* from diseased seedlings using PCR. The sequence of the ITS region of *P. ultimum* was identical with that of *Pythium* group HS. The results support the reports that the HS group is an asexual strain of *P. ultimum*. Using PCR, the primer pair K1+K3, designed on portions of the sequence of the ITS region, amplified isolates of *P. ultimum* and the HS group but not isolates of 20 other *Pythium* species. DNA extracts from damped-off seedlings were not amplified, but a 10-fold dilution of the extracts with Tris-EDTA (TE) buffer diluted the inhibitors and allowed PCR amplification. The primer pair used detected *P. ultimum* from a single diseased seedling.

The genus *Pythium* consists of more than 120 species (11). The identification keys provided by Plaats-Niterink (34) and Dick (11) are commonly used by many workers. The major structural features, such as the size of oogonia and oospores, the morphology of oogonia, antheridia, and sporangia, and the growth habit and rate on culture medium can be characterized. However, it is difficult to determine the detailed features, such as antheridial branch origin, antheridial attachment, and plerotic or aplerotic status of oospores, which vary under different cultural conditions (16). Therefore, the identification of some *Pythium* species is very difficult. The use of a selective medium is well established for isolating many species from soil or diseased tissue (1,18,21). However, if the population density of a specific species is to be estimated, the colonies that appear on the selective medium must be individually transferred to culture media for identification. Problems of identification and quantification have limited the study of the ecology of *Pythium* species and the diagnosis of species responsible for diseases.

DNA probes have been developed for identification of *Pythium* species (22,26,30).

The polymerase chain reaction (PCR) for amplification of diagnostic molecular markers is highly sensitive and reproducible, and could easily be used for identification and detection if species-specific primers were designed. Such a technique has been used to identify fungi, including *Acremonium coenophialum* (12), *Fusarium* spp. (37), *Gaeumannomyces graminis* (17), *Monosporascus* spp. (28), *Septoria tritici* and *Stagonospora nodorum* (4), and *Verticillium* spp. (31,32). Nuclear rDNA consists of the small and large subunits, 5.8S, and the internal transcribed spacer (ITS) region in fungi, and each sequence is variable at the family, genus, or species level. In *Pythium*, the ITS region is variable at the species level (6-8). The number of copies of the rDNA repeat is usually more than 50 per genome, suggesting a possible high sensitivity for detection if used as a target gene for PCR. Thus, the ITS region of rDNA may be valuable for detection of individual species of *Pythium* using PCR amplification.

Pythium ultimum Trow is a widespread and important plant pathogen causing damping-off and root rot in many plant species (15). The HS group, according to the identification key of Plaats-Niterink (34), is reported to be an asexual strain of *P. ultimum* (13,29,36).

In this study, we report the sequence of the ITS region of the rDNA of *P. ultimum* and group HS, the design of species-specific primers for PCR, and a procedure for detection of *P. ultimum* from diseased plants using PCR.

MATERIALS AND METHODS

Isolates and DNA extraction from mycelia. Isolates of *Pythium* used in this study

are listed in Table 1. The cultures were maintained on cornmeal agar at 25°C. For DNA extraction, three agar plugs were removed from the growing margin of 2-day-old cultures on potato carrot agar with a 1-cm cork borer and transferred to 20% V8 juice broth containing 2.5 g of CaCO₃ per liter. Mycelial mats were collected on filter paper placed in a Buchner funnel after a 3- to 4-day incubation period at 25°C and rinsed several times with distilled water. After excess water was removed with filter paper, the mycelial mats were frozen at -80°C for more than 1 day and powdered by using a sterilized, prefrozen mortar and pestle. The extraction of DNA was conducted according to the procedure of Lee and Taylor (24), except for precipitating DNA with 99.8% cold ethanol. The DNA was dissolved in 200 µl of TE buffer (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA).

PCR procedure. The universal and the designed primers in the present study (Table 2) were used to amplify the ITS region as described by White et al. (41). A total volume of 25 µl of reaction mixture contained 1 µM of each primer, 1.25 units of *rTaq* DNA polymerase (Takara Shuzo Co. Ltd., Shiga, Japan), 0.2 mM dNTP mixture, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl₂), and 100 to 300 ng of DNA template. The reaction was conducted using a DNA thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT), and the amplification condition was an initial denaturation at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min with final extension at 72°C for 10 min. PCR products were precipitated with cold ethanol, and their size was examined by electrophoresis for 60 min in 1.2% Agarose LO3 (Takara Shuzo Co. Ltd., Shiga, Japan) gel. Gels were stained with ethidium bromide and photographed under ultraviolet light.

Sequencing PCR products. The amplified ITS fragments from *P. ultimum*, Py79, and the HS group, Py55, were cloned using the TA Cloning Kit and pCRII vector (Invitrogen Corporation, San Diego, CA). The DNA sequences of the ITS regions were determined by the method of Sanger et al. (35) using an automated sequencer (Applied Biosystems, model 373A, Foster City, CA). DNA sequences were analyzed

Corresponding author: K. Kageyama
E-mail: kageyama@cc.gifu-u.ac.jp

Current address of A. Ohya: Plant Breeding and Genetics Research Institute, Japan Tobacco Inc., Iwata, Shizuoka 438, Japan

Accepted for publication 17 June 1997.

using a software from GENETYX (Software Development Co. Ltd., Tokyo, Japan).

DNA extraction from damped-off seedlings. Three plant species, Chinese cabbage (*Brassica campestris* var. *chinesis* (L.) Makino cv. Muso), cucumber (*Cucumis sativus* L. cv. Jibai), and sugar beet (*Beta vulgaris* L. var. *saccharifera* Alef. cv. Monoace), were grown either in a potting soil mix (Starbed, Kyodouhiryo Co. Ltd., Japan) infested with a mycelial suspension of *P. ultimum* as described by Kageyama and Ui (20), or in soil naturally infested with *P. ultimum* as well as several other *Pythium* species. The naturally infested soil was collected from a Chinese cabbage field in Gifu, Japan. Twenty seeds were planted in pots (5 cm diameter) and were incubated in a growth chamber at 20

to 25°C. Seedlings showing damping-off symptoms were immediately taken from the pots. Healthy plants were collected 2 weeks after seeding. A water-soaked lesion of a damped-off seedling and a hypocotyl of a healthy seedling were cut into two sections and used for DNA extraction and for isolation of *Pythium* species, respectively, after washing the diseased seedling under tap water. The preparation of the specimens before DNA extraction was done using two methods: (i) macerating the specimens frozen at -80°C with a sterilized, prefrozen mortar and pestle; and (ii) cutting the specimens into approximately 1-mm pieces. DNA was extracted using the DNA extraction kit (ISOPLANT, Nippon Gene Co. Ltd., Toyama, Japan), which had benzyl chloride as a main component, following the manufacturer's instructions.

The DNA was dissolved in 20 µl of TE buffer and diluted 0- to 100-fold with TE buffer before PCR amplification.

Southern hybridization. PCR products were separated by electrophoresis in a 1.5% agarose gel and transferred onto a nucleic acid transfer nylon membrane (Hybond N+, Amersham International plc, Amersham, UK) by alkaline transfer in 0.4 M NaOH. A hybridization probe was produced from the PCR product of *P. ultimum*, Py79 isolate, and labeled with digoxigenin (Boehringer Mannheim, Indianapolis, IN). The hybridization and detection was performed with the Dig nucleic acid detection kit (Boehringer Mannheim). Both procedures were done according to the manufacturer's instruction.

Isolation of *P. ultimum* from damped-off plants. A portion of the water-soaked

Table 1. Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of *Pythium* species using universal and species-specific primers

Species	Isolates	Host/habitat	Geographic origin	PCR products	
				ITS ^a	SS ^b
<i>P. ultimum</i>	MK2-2-1V	Bent grass green soil	Gifu, Japan	+	+
<i>P. ultimum</i>	OF231	Carrot field soil	Gifu, Japan	+	+
<i>P. ultimum</i>	Py 79	Sugar beet	Hokkaido, Japan	+	+
<i>P. ultimum</i>	UOP358	Chinese cabbage	Sendai, Japan	+	+
Group HS	BeBS 2	Kidney bean field soil	Hokkaido, Japan	+	+
Group HS	Py 55	Sugar beet	Hokkaido, Japan	+	+
Group HS	SoyBS 13	Soybean field soil	Hokkaido, Japan	+	+
Group HS	18-3	Carrot field soil	Gifu, Japan	+	+
<i>P. aphanidermatum</i>	UOP390	Spinach	Osaka, Japan	+	-
<i>P. aphanidermatum</i>	TOc159	Carrot field soil	Gifu, Japan	+	-
<i>P. arrhenomanes</i>	GUP6	Sugarcane	Kagoshima, Japan	+	-
<i>P. arrhenomanes</i>	GUP7	Sugarcane	Kagoshima, Japan	+	-
<i>P. catenulatum</i>	ATCC10950			+	-
<i>P. catenulatum</i>	MK4-10-2S	Zoysia grass	Gifu, Japan	+	-
<i>P. chamaeophyphon</i>	MS6-10-8V	Forest soil	Gifu, Japan	+	-
<i>P. coloratum</i>	TM321	Carrot	Gifu, Japan	+	-
<i>P. coloratum</i>	Z712	Carrot	Gifu, Japan	+	-
<i>P. graminicola</i>	32-1 sh	Zoysia grass	Japan	+	-
<i>P. graminicola</i>	TS2	Sugarcane	Kagoshima, Gifu	+	-
<i>P. irregulare</i>	Py 66	Sugar beet	Hokkaido, Japan	+	-
<i>P. irregulare</i>	6K4SSDP	Zoysia grass coarse soil	Gifu, Japan	+	-
<i>P. iwayamai</i>	MK1-2-18V	Zoysia grass coarse soil	Gifu, Japan	+	-
<i>P. iwayamai</i>	MK4-6-5S	Zoysia grass	Gifu, Japan	+	-
<i>P. myriotylum</i>	BeAT1	Kidney bean	Hokkaido, Japan	+	-
<i>P. myriotylum</i>	BeBT1	Kidney bean	Hokkaido, Japan	+	-
<i>P. orthogonon</i>	DS2-6-9D	Zoysia grass	Gifu, Japan	+	-
<i>P. orthogonon</i>	MS1-9-2S	Zoysia grass	Gifu, Japan	+	-
<i>P. paroecandrum</i>	ATCC36784	Barley	United States	+	-
<i>P. paroecandrum</i>	BeA-7	Kidney bean	Hokkaido, Japan	+	-
<i>P. peritum</i>	MK4-8-11V	Zoysia grass coarse soil	Gifu, Japan	+	-
<i>P. peritum</i>	S2-8-1S	Zoysia grass	Gifu, Japan	+	-
<i>P. periplocum</i>	DS1-7-2S	Zoysia grass	Gifu, Japan	+	-
<i>P. periplocum</i>	DK1-5-1S	Zoysia grass	Gifu, Japan	+	-
<i>P. rostratum</i>	DS5-7-1S	Bent grass	Gifu, Japan	+	-
<i>P. rostratum</i>	MK2-1-9S	Bent grass	Gifu, Japan	+	-
<i>P. spinosum</i>	Py 75	Sugar beet	Hokkaido, Japan	+	-
<i>P. spinosum</i>	TN254	Carrot	Gifu, Japan	+	-
<i>P. splendens</i>	ATCC42852	<i>Luculia gratissima</i>	New Zealand	+	-
<i>P. sulcatum</i>	CTMa7	Carrot	Gifu, Japan	+	-
<i>P. sulcatum</i>	GUP2	Carrot	Gifu, Japan	+	-
<i>P. sylvaticum</i>	Py 35 Ho-M	Sugar beet	Hokkaido, Japan	+	-
<i>P. sylvaticum</i>	S66-4-8	Kidney bean	Hokkaido, Japan	+	-
<i>P. torulosum</i>	S2-1 sz-1	Zoysia grass	Kyoto, Japan	+	-
<i>P. torulosum</i>	TJu143	Carrot	Gifu, Japan	+	-
<i>P. vanterpoolii</i>	DK1-6-3D	Zoysia grass coarse soil	Gifu, Japan	+	-
<i>P. vanterpoolii</i>	UOP392	Zoysia grass	Japan	+	-

^a Universal primer set, ITS1+ITS4, was used. The + indicates that PCR product (910 bp) is obtained.

^b Potential species-specific primer set, K1+K3, was used. The + indicates that PCR product (670 bp) is obtained.

lesion left after DNA extraction was directly plated on a *Pythium* selective medium (19) consisting of 17 g of Difco cornmeal agar, 5 mg of pimaricin, 100 mg of pentachloronitrobenzene, and 100 mg of agrimycin per liter. Plates were incubated at 25°C in the dark for 2 to 6 days. Isolates were identified according to the key of Plaats-Niterink (34).

RESULTS

Sequence of the ITS region of *P. ultimum*. The sequence of the ITS region of *P. ultimum* (Py79) was identical with that of the isolate of HS group (Py55). PCR products consisted of 3' ends of 18S rDNA, ITSa region, 5.8S rDNA, ITSb region, and 5' ends of 28S rDNA with 914 bp (Fig. 1).

Primer design and specificity to *P. ultimum*. The three primers shown in Figure 1 and Table 2 were synthesized and designated K1, K2, and K3. Because the ITSa has interspecific differences in *Pythium* species and the center of the ITSb is not species-specific (26), the primers were designed in the center of the ITSa and downstream of the ITSb or the 5' ends of 28S rDNA. The six possible combinations of the primers, K1+K2, K1+K3, K1+ITS4, ITS1+K2, ITS1+K3, and ITS1+ITS4, were compared to examine the specificity to *P. ultimum* (Py79), HS group (Py55), *P. syl-*

vaticum (Py35), and *P. irregulare* (Py61). Four primer combinations, K1+K2, K1+K3, K1+ITS4, and ITS1+K3, did not produce PCR products for *P. sylvaticum* and *P. irregulare*. In *P. ultimum* and HS group isolates, the ITS region was amplified with all primer combinations, suggesting that K1 and K3 may be species-specific.

A total of 46 isolates representing 21 *Pythium* species and the HS group were used to test the species-specificity of primer combination K1+K3 to *P. ultimum* (Table 1). PCR products, 910 and 670 bp, were obtained in each combination of universal primers (ITS1+ITS4) and potential specific primers (K1+K3), respectively, for four isolates each of *P. ultimum* and the HS group obtained from different host plants or soils collected in geographically different locations in Japan. However, for the isolates representing the other species, PCR amplification products (910 bp) were found only for the primer combination, ITS1+ITS4.

Detection of *P. ultimum* in DNA extracts from damped-off seedlings using PCR amplification. DNA was extracted from five damped-off seedlings each of cucumber, sugar beet, and Chinese cabbage grown in soil artificially infested with *P. ultimum*. The hypocotyls with roots were

frozen at -80°C and then macerated with a sterilized, prefrozen mortar and pestle. PCR products (670 bp) from initial DNA extracts were not detected by electrophoresis or Southern hybridization, except in cucumber (Fig. 2). However, when the DNA extracts were diluted 10- and even 100-fold with TE buffer, distinct bands for the pathogen were observed for cucumber, sugar beet, and Chinese cabbage. The DNA extracts were diluted 10-fold with TE buffer in subsequent experiments. PCR amplification was performed twice for each DNA extract, and the same results were obtained.

Plants of each of the three species showing damped-off symptoms in artificially infested soil were prepared by macerating one or five frozen lesions (-80°C) with a sterilized, prefrozen mortar and pestle or by cutting one lesion into approximately 1-mm pieces. The bands (670 bp) were observed from the DNA extracts,

Table 2. Primer sequence

Primer	Sequence
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC
K1	ACGAAGGTTGGTCTGTTG
K2	TCTTGTCTGATATCAGGTC
K3	TCTCTACGCACTAAATGC

Fig. 1. Sequence of the rDNA internal transcribed spacer (ITS) region of *Pythium ultimum* isolate Py79 and *Pythium* group HS isolate Py55. The sequences were completely identical for the two isolates. The amplified DNA consists of 3' end of 18S rDNA, ITSa, 5.8S rDNA, ITSb, and 5' end of 28S rDNA. ITS1 and ITS 4 are universal primers, and K1, K2, and K3 are selected as primers in the present experiments. The accession number of the DDBJ databases is D86515.

both from a single seedling and from five damped-off seedlings of all plant species, by means of Southern hybridization, but not from a single seedling of Chinese cabbage for electrophoresis. When preparation methods of the specimens before DNA extraction were compared, the bands from PCR products amplified from diluted DNA extracts were observed in Southern hybridization regardless of the preparation method used (Fig. 3). PCR amplification was performed twice for each DNA extract with the same results.

Ten damped-off seedlings each of cucumber, sugar beet, and Chinese cabbage in naturally infested soil were used to compare detection of *P. ultimum* by isolation from lesions on selective medium and PCR with the species-specific primers (Table 3). In three to five repeated experiments, the disease incidence averaged 6.5, 11.9, and 19.2% for cucumber, sugar beet, and Chinese cabbage, respectively. For DNA extraction, the specimens were prepared by cutting one lesion to approximately 1 mm in length. In cucumber, *P. ultimum* was recovered from 10 seedlings, and the bands (670 bp) were observed from all of the diseased seedlings in Southern hybridization, although not from a single seedling in electrophoresis. In sugar beet, amplification was observed in seven damped-off seedlings from which *P. ultimum* was isolated, in one seedling from which another *Pythium* species was isolated, and in one seedling from which no *Pythium* was isolated in either electrophoresis or Southern hybridization. In Chinese cabbage, the bands in electrophoresis were observed in four diseased seedlings from which *P. ultimum* was isolated, while the signals in Southern hybridization were obtained in all of five damped-off seedlings from which *P. ultimum* was isolated and in a single seedling from which another *Pythium* species was obtained. No amplification occurred when DNA extracts from healthy plants of three plant species were used. PCR amplification was performed twice for each DNA extract with the same results.

DISCUSSION

The ITS region of the rDNA is highly conserved within a fungal species but not between species (41). With *Pythium* species, restriction fragment length polymorphism (RFLP) analysis has been used to distinguish species (6,8). In the present study, the sequence of the ITS region of *P. ultimum* was identical with that of the HS group. This supports other lines of evidence that the HS group is an asexual strain of *P. ultimum* that includes restriction patterns of mitochondrial DNA (29) and the ability to outcross (13,36).

Information is available on DNA sequences of the ITS region in fungi. These include sequencing the region for *Phytophthora* spp. (25), *Fusarium sambucinum*

Fig. 2. Polymerase chain reaction (PCR) amplification of diluted and nondiluted DNA extracts from seedlings exhibiting damping-off symptoms in soil artificially infested with *Pythium ultimum* using species-specific primers. Seedlings of cucumber (Cuc), sugar beet (Sug), and Chinese cabbage (Chi) were used. The DNA extracts were not (1), 10-fold (10), and 100-fold (100) diluted with TE buffer. Lane M: size marker from *Sst*I digestion of bacteriophage lambda. (A) Agarose gel electrophoresis; (B) Southern hybridization using the digoxigenin-labeled probe from the PCR product of *P. ultimum*.

(33), *Metarhizium* spp. (9), *Colletotrichum* spp. (38,40), *Gaeumannomyces graminis* (14), and *Septoria tritici* and *Stagonospora nodorum* (4). The sequence of the region described in the present study is an initial finding in the genus *Pythium*. The restriction sites based on the sequence of the ITS region were similar to those described by Levesque et al. (26) and Chen et al. (8), suggesting that the isolates used in the present study would be closely related to isolates from other parts of the world. The determined sequence showed little homology with previously identified sequences of other fungi registered in the DDBJ and GeneBank databases. The nucleotide sequence data reported in this paper will appear in the DDBJ database under accession number D86515.

Few studies have reported the detection of a pathogen from naturally infected plants (5). This might be due to inhibitors to PCR amplification such as humic acids (23). Hypocotyls and roots showing rot

Fig. 3. The effect of preparation methods of damped-off seedling materials for DNA extraction on detection of *Pythium ultimum* by polymerase chain reaction (PCR) with species-specific primers. The seedlings of cucumber (Cuc), sugar beet (Sug), and Chinese cabbage (Chi) were collected from soil artificially infested with *P. ultimum*. The preparation of the seedlings was conducted by macerating five (lane 1) and one (lane 2) seedlings frozen at -80°C with a sterilized, prefrozen mortar and pestle, respectively, and by cutting one seedling into approximately 1-mm pieces (lane 3). Lane M: the size marker from *Sst*I digestion of bacteriophage lambda. (A) Agarose gel electrophoresis; (B) Southern hybridization using the digoxigenin-labeled probe from the PCR product of *P. ultimum*.

symptoms collected from soil will potentially contain humic acids, and in this study these portions were used for DNA extraction. Initial attempts to amplify the ITS region of the rDNA from diseased seedlings suggested the presence of inhibitors. However, PCR products were detected from seedlings naturally infected with *P. ultimum* following dilution of the extracted DNA with TE buffer, suggesting that dilution can overcome the effects of inhibitors.

Immunological techniques have also been developed to detect plant pathogens (2,3,42). Monoclonal antibodies recognize a single epitope on a protein; therefore its specificity is as high as that of the PCR technique using specific primers (10,27,39). However, if a researcher should decide to conduct similar experiments, the

Table 3. Detection of *Pythium ultimum* from damped-off seedlings in naturally infested soil by polymerase chain reaction (PCR) with species-specific primers

Damped-off seedlings	Isolation of <i>Pythium</i> ^a	Bands ^b	
		Electrophoresis	Southern blotting ^c
Cucumber			
Healthy	...	—	—
1	<i>P. ultimum</i>	+	+
2	<i>P. ultimum</i>	+	+
3	<i>P. ultimum</i>	+	+
4	<i>P. ultimum</i>	+	+
5	<i>P. ultimum</i>	+	+
6	<i>P. ultimum</i>	+	+
7	<i>P. ultimum</i>	+	+
8	<i>P. ultimum</i>	+	+
9	<i>P. ultimum</i>	—	+
10	<i>P. ultimum</i>	+	+
Sugar beet			
Healthy	...	—	—
1	<i>P. ultimum</i>	+	+
2	<i>P. ultimum</i>	+	+
3	<i>P. ultimum</i>	+	+
4	<i>P. ultimum</i>	+	+
5	<i>P. ultimum</i>	+	+
6	<i>P. ultimum</i>	+	+
7	<i>P. ultimum</i>	+	+
8	<i>Pythium</i> sp.	—	—
9	<i>Pythium</i> sp.	+	+
10	...	+	+
Chinese cabbage			
Healthy	...	—	—
1	<i>P. ultimum</i>	+	+
2	<i>P. ultimum</i>	+	+
3	<i>P. ultimum</i>	+	+
4	<i>P. ultimum</i>	+	+
5	<i>P. ultimum</i>	—	+
6	<i>Pythium</i> sp.	—	+
7	<i>Pythium</i> sp.	—	—
8	<i>Pythium</i> sp.	—	—
9	<i>Pythium</i> sp.	—	—
10	<i>Pythium</i> sp.	—	—

^a A portion of the lesion used for DNA extraction was plated on *Pythium* selective medium. The colony was transferred and identified.

^b The probe was produced by labeling the PCR products from *P. ultimum* with digoxigenin-11-dUTP.

^c The size of the band was 670 bp.

antibody must be provided by the original researcher. In PCR, if the primer sequences are available, the primers can be synthesized independently, and the same method could be followed by any researcher.

In the present study, the combination of the primers, K1+K3, is species-specific to *P. ultimum* and will selectively amplify DNA from isolates of *P. ultimum* and HS group but not from other isolates of *Pythium* species. In addition, the use of the primer pair could detect *P. ultimum* from single diseased seedlings. Further study is needed to develop a technique to detect *P. ultimum* in soil.

P. ultimum was detected by PCR amplification in samples from which no *Pythium* species or other *Pythium* species were isolated on the selective medium for sugar beet and Chinese cabbage, respectively. The presence and growth of other *Pythium* species or microorganisms may interfere with the isolation of *P. ultimum* on the media. In addition, the results suggest that isolation on a selective medium is less sensitive than the PCR technique. However, the bands may correspond to a non-

specific reaction of the primers, although the size of the PCR products was identical with that from *P. ultimum*. Further experiments will be needed to verify the value of these species-specific primers by using additional isolates of other *Pythium* species.

ACKNOWLEDGMENTS

We thank T. Ichitani for supplying the isolates of *Pythium aphanidermatum*, *P. ultimum*, and *P. vanterpoolii*.

LITERATURE CITED

1. Ali-Shtayeh, M. S., Len, L. C., and Dick, M. W. 1986. An improved method and medium for quantitative estimates of populations of *Pythium* species from soil. Trans. Br. Mycol. Soc. 86:39-47.
2. Amouzou-Alladaye, E., Dunez, J., and Clerjeau, M. 1988. Immunoenzymatic detection of *Phytophthora fragariae* in infected strawberry plants. Phytopathology 78:1022-1026.
3. Avila, F. J., Yuen, G. Y., and Klopfenstein, N. B. 1995. Characterization of a *Pythium ultimum*-specific antigen and factors that affect its detection using a monoclonal antibody. Phytopathology 85:1378-1387.
4. Beck, J. J., and Ligon, J. M. 1995. Polymerase chain reaction assays for the detection of *Stagonospora nodorum* and *Septoria*

- tritici* in wheat. Phytopathology 85:319-324.
5. Braithwaite, K. S., Egeskov, N. M., and Smith, G. R. 1995. Detection of sugarcane bacilliform virus using the polymerase chain reaction. Plant Dis. 79:792-796.
6. Chen, W. 1992. Restriction fragment length polymorphisms in enzymatically amplified ribosomal DNAs of three heterothallic *Pythium* species. Phytopathology 82:1467-1472.
7. Chen, W., and Hoy, J. W. 1993. Molecular and morphological comparison of *Pythium arrhenomanes* and *P. graminicola*. Mycol. Res. 97:1371-1378.
8. Chen, W., Hoy, J. W., and Schneider, R. W. 1992. Species-specific polymorphisms in transcribed ribosomal DNA of five *Pythium* species. Exp. Mycol. 16:22-34.
9. Curran, J., Driver, F., Ballard, J. W. O., and Milner, R. J. 1994. Phylogeny of *Metarhizium*: Analysis of ribosomal DNA sequence data. Mycol. Res. 98:547-552.
10. De Boer, S. H., and Ward, L. J. 1995. PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. Phytopathology 85:854-858.
11. Dick, M. W. 1990. Key to *Pythium*. Department of Botany, University of Reading, Reading, UK. pp. 1-64.
12. Doss, R. P., and Welty, R. E. 1995. A polymerase chain reaction-based procedure for detection of *Acremonium coenophialum* in tall fescue. Phytopathology 85:913-917.
13. Francis, D. M., and St. Clair, D. A. 1993. Outcrossing in the homothallic oomycete, *Pythium ultimum*, detected with molecular markers. Curr. Genet. 24:100-106.
14. Goodwin, P. H., Hsiang, T., Xue, B. G., and Liu, H. W. 1995. Differentiation of *Gaeumannomyces graminis* from other turf-grass fungi by amplification with primers from ribosomal internal transcribed spacer. Plant Pathol. 44:384-391.
15. Hendrix, F. F., Jr., and Campbell, W. A. 1973. *Pythiums* as plant pathogens. Annu. Rev. Phytopathol. 11:77-98.
16. Hendrix, F. F., Jr., and Papa, K. E. 1974. Taxonomy and genetics of *Pythium*. Proc. Am. Phytopathol. Soc. 1:200-207.
17. Henson, J. M., Goins, T., Grey, W., Mathre, D. E., and Elliott, M. L. 1993. Use of polymerase chain reaction to detect *Gaeumannomyces graminis* DNA in plants grown in artificially and naturally infested soil. Phytopathology 83:283-287.
18. Kageyama, K., Tachi, M., Umetsu, M., and Hyakumachi, M. 1996. Epidemiology of *Pythium sulcatum* associated with brown-blotted root rot of carrots. Ann. Phytopathol. Soc. Jpn. 62:130-133.
19. Kageyama, K., and Ui, T. 1980. A selective medium for isolation of *Pythium* spp. Ann. Phytopathol. Soc. Jpn. 46:542-544.
20. Kageyama, K., and Ui, T. 1981. *Pythium* species isolated from bean plants and soils in the experimental plots of monoculture and rotation. Ann. Phytopathol. Soc. Jpn. 47:313-319.
21. Kageyama, K., Ui, T., and Narita, Y. 1981. Influence of *Pythium* spp. on the injury by bean monoculture. Ann. Phytopathol. Soc. Jpn. 47:320-326.
22. Klassen, G. R., Balcerzak, M., and de Cock, A. W. A. M. 1996. 5S ribosomal RNA gene spacers as species-specific probes for eight species of *Pythium*. Phytopathology 86:581-587.
23. Kreader, C. A. 1996. Relief of amplification inhibition in PCR with bovine serum albumin of T4 gene 32 protein. Appl. Environ. Microbiol. 62:1102-1106.
24. Lee, S. B., and Taylor, J. W. 1990. Isolation of DNA from fungal mycelia and single spores. Pages 282-287 in: PCR Protocols: A Guide to

- Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, New York.
25. Lee, S. B., and Taylor, J. W. 1992. Phylogeny of five fungus-like protistans *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Mol. Biol. Evol.* 9:636-653.
 26. Levesque, A. C., Vrain, C. T., and De Boer, S. H. 1994. Development of a species-specific probe for *Pythium ultimum* using amplified ribosomal DNA. *Phytopathology* 84:474-478.
 27. Li, X., and De Boer, S. H. 1995. Selection of polymerase chain reaction primers from an RNA intergenic spacer region for specific detection of *Clavibacter michiganensis* subsp. *sepedonicus*. *Phytopathology* 85:837-842.
 28. Lovic, B. R., Martyn, R. D., and Miller, M. E. 1995. Sequence analysis of the ITS region of rDNA in *Monosporascus* spp. to evaluate its potential for PCR-mediated detection. *Phytopathology* 85:655-661.
 29. Martin, F. N. 1990. Taxonomic classification of asexual isolates of *Pythium ultimum* based on cultural characteristics and mitochondrial DNA restriction patterns. *Exp. Mycol.* 14:47-56.
 30. Martin, F. N. 1991. Selection of DNA probes useful for isolate identification of two *Pythium* spp. *Phytopathology* 81:742-746.
 31. Moukhamedov, R., Hu, X., Nazar, R. N., and Robb, J. 1994. Use of polymerase chain reaction-amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricorpus*. *Phytopathology* 84:256-259.
 32. Nazar, R. N., Hu, X., Schmidt, J., Culham, D., and Robb, J. 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.
 33. O'Donnell, K. 1992. Ribosomal DNA internal transcribed spacer are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Curr. Genet.* 22:213-220.
 34. Plaats-Niterink, A. J. van der. 1981. Monograph of the genus *Pythium*. Studies in Mycology, No. 21. Centraalbureau Voor Schimmelcultures, Baarn, Netherlands.
 35. Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463-5467.
 36. Saunders, G. A., and Hancock, J. G. 1994. Self-sterile isolates of *Pythium* mate with self-fertile isolates of *Pythium ultimum*. *Mycologia* 86:660-666.
 37. Schilling, A. G., Moller, E. M., and Geiger, H. H. 1996. Polymerase chain reaction-based assay for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. *Phytopathology* 86:515-522.
 38. Sherriff, C., Whelan, M. J., Arnold, G. M., and Bailey, J. A. 1995. rDNA sequence analysis confirms the distinction between *Colletotrichum graminicola* and *C. sublineolum*. *Mycol. Res.* 99:475-478.
 39. Slack, S. A., Drennan, J. L., Westra, A. A. G., Gudmestad, N. C., and Oleson, A. E. 1996. Comparison of PCR, ELISA, and DNA hybridization for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. *Plant Dis.* 80:519-524.
 40. Sreenivasaprasad, S., Mills, P. R., and Brown, A. E. 1994. Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. acutatum*. *Mycol. Res.* 98:186-188.
 41. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, New York.
 42. Yuen, G. Y., Craig, M. L., and Avila, F. 1993. Detection of *Pythium ultimum* with a species-specific monoclonal antibody. *Plant Dis.* 77:692-698.