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PCR Amplification of Species-Specific DNA Sequences Can Distinguish among *Phytophthora* Species†

T. ÉRSEK,‡ J. E. SCHOELZ, AND J. T. ENGLISH*

Department of Plant Pathology, University of Missouri, Columbia, Missouri, 65211

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We used PCR to differentiate species in the genus *Phytophthora*, which contains a group of devastating plant pathogenic fungi. We focused on *Phytophthora parasitica*, a species that can infect solanaceous plants such as tomato, and on *Phytophthora citrophthora*, which is primarily a citrus pathogen. Oligonucleotide primers were derived from sequences of a 1,300-bp *P. parasitica*-specific DNA segment and of an 800-bp *P. citrophthora*-specific segment. Under optimal conditions, the primers developed for *P. parasitica* specifically amplified a 1,000-bp sequence of DNA from isolates of *P. parasitica*. Primers for *P. citrophthora* similarly and specifically amplified a 650-bp sequence of DNA from isolates of *P. citrophthora*. Detectable amplification of these specific DNA sequences required picogram quantities of chromosomal DNA. Neither pair of primers amplified these sequences with DNAs from other species of *Phytophthora* or from the related genus *Pythium*. DNAs from *P. parasitica* and *P. citrophthora* growing in infected tomato stem tissue were amplified as distinctly as DNAs from axenic cultures of each fungal species. This is the first report on PCR-driven amplification with *Phytophthora* species-specific primers.

The genera *Phytophthora* and *Pythium* and other genera of *Oomycete* fungi appear to be related more closely to algae than to other groups of fungi (6). Many members of these genera have proven recalcitrant to attempts to develop genetic marker systems (23). Recently, drug resistance markers have been established for *Phytophthora* spp. by either chemical mutagenesis (2, 15, 17, 18, 32) or transformation (1, 14). Species-specific oligonucleotide probes that complement different rDNA internal transcribed spacer (ITS) sequences have been reported for four *Phytophthora* spp. (16). Furthermore, species-specific repetitive sequences have been isolated from chromosomal DNA of *Phytophthora parasitica* Dastur and *Phytophthora citrophthora* (Sm. & Sm.) Leonian (7, 9, 10). Such specific cloned DNA fragments have been used as hybridization probes for the detection and identification of *Phytophthora* species from cultures and from plant and soil samples, and for examination of genetic variation among populations by restriction fragment length polymorphisms (8, 10).

In this report we describe the development of genetic markers for *P. parasitica* and *P. citrophthora* that are based on PCR. The availability of species-specific fragments as potential genetic markers from chromosomal DNAs of *P. parasitica* and *P. citrophthora* suggested that oligonucleotide primer sequences derived from each fragment could be used to amplify the corresponding template sequences by means of PCR (12, 27, 28). The PCR products of each species would be unique and thus should prove useful for the detection of putative interactions (e.g., genetic exchange) among concomitantly infecting species. The great advantage of PCR over techniques such as DNA-DNA hybridization lies in the requirement for only small amounts of target DNA and in the rapidity of the procedure. The procedure has been applied successfully in phytopathological research to detect plant pathogenic fungi in host tissues directly (5, 13, 26, 30, 33) and to isolate a fungal gene sequence relevant in terpenoid biosynthesis (4).

The development of genetic markers with a PCR system is the first step in elaborating a tracking system for the evaluation of population dynamics at both organismal and genetic levels among multiple *Phytophthora* spp. in concomitantly infected plants. The host-parasite system being studied includes tomato (*Lycopersicon esculentum* L.) and three *Phytophthora* spp.: *P. parasitica* and *Phytophthora capsici* Leonian, both of which are extremely pathogenic on many plants including tomato, and *P. citrophthora*, which is primarily a citrus pathogen but also can be weakly pathogenic on tomato, under laboratory conditions.

MATERIALS AND METHODS

Fungal isolates. Isolates of *Phytophthora* and *Pythium* spp. and their sources are listed in Table 1. Fungal cultures were maintained on cornmeal (Difco Laboratories, Detroit, Mich.) slants and were stored at 16°C.

DNA isolation. Chromosomal DNA was extracted from lyophilized mycelium and was purified according to the method of Goodwin et al. (9). Genomic DNAs of *P. parasitica*, *P. citrophthora*, and *P. capsici* also were extracted from tomato stem tissue colonized with each *Phytophthora* sp. Stems of 18-day-old plants (cv. Peto 343; Petoseed Co., Inc., Woodland, Calif.) were wounded halfway between the soil level and the cotyledonary leaves and were inoculated with a 2-mm agar plug colonized by mycelium of one of the mentioned species. Control inoculations were made with sterile agar plugs. Two to seven days after inoculation, 5-mm-long stem pieces were excised from below and above the approximately 5-mm vertical wound. Between 0.5 and 1 g of stem tissues thus obtained was frozen immediately in liquid nitrogen and were ground to a fine powder. A "miniprep" procedure described for the extraction of total *Phytophthora* sp. DNA (9) was then used, with slight modification. Powdered samples were extracted with buffer (100 mM Tris, 40 mM EDTA, 0.2% sodium dodecyl sulfate [SDS], and 0.2% 2-mercaptoethanol, pH 8.0) for 30 min on ice and were centrifuged at 12,000 × *g* for 10 min. The

* Corresponding author. Mailing address: 108 Waters Hall, Department of Plant Pathology, University of Missouri, Columbia, MO 65211. Phone: (314) 882-1472. Fax: (314) 882-0588.

† Missouri Agricultural Experiment Station Journal Series No. 12,126.

‡ Permanent address: Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary.

TABLE 1. Species-specific amplification of DNA sequences from isolates of *Phytophthora* and *Pythium* species with oligonucleotide primers derived from *P. parasitica* 5-3A (Pp) and *P. citrophthora* P1323 (Pci)

Phytophthora or *Pythium* isolate	Source	Amplification[a] obtained with primers derived from:	
		Pp	Pci
Phytophthora parasitica			
5-3A	Tomato, California[b]	+	−
W1	Tomato, California[b]	+	−
C-2CL	Citrus, Arizona[c]	+	−
Mis.-22C	Jojoba, Arizona[c]	+	−
P. citrophthora			
P1213	Cacao, Brazil[d]	−	+
P1201	Cacao, Brazil[d]	−	+
P449	Cacao, Brazil[d]	−	+
32-4-7	Walnut, Chile[e]	−	+
18-4-8	Walnut, Chile[e]	−	+
34-4-5	Cherry, California[e]	−	+
P1323	Citrus, California[b]	−	+
P. capsici			
1787	Soil, California[f]	−	−
1794	Soil, California[f]	−	−
P. palmivora			
P6594	Soil/citrus, Florida[d]	−	−
P7655	Coconut palm, Indonesia[d]	−	−
P. megasperma			
P6519	Sweet almond, California[d]	−	−
P7707	Alfalfa, Wisconsin[d]	−	−
P. infestans			
P1306	Tomato, California[d]	−	−
P7913	Tomato, California[d]	−	
P. citricola			
SG3-20	River, California[e]	−	−
P. cambivora			
12-4-5	Apple, California[e]	−	−
P. erythroseptica			
PO-1	Potato, California[d]	−	−
P. cryptogea			
RI	Safflower, Arizona[b]	−	−
Pythium ultimum			
67-1	Cotton, California[g]	−	−
P. irregulare			
86-10	Alfalfa, California[g]	−	−

[a] +, present; −, absent.
[b] Obtained from the culture collection of J. M. Duniway, Department of Plant Pathology, University of California, Davis.
[c] Obtained from the culture collection of M. E. Matheron, Yuma Mesa Agricultural Center, Somerton, Ariz.
[d] Obtained from the culture collection of M. D. Coffey, Department of Plant Pathology, University of California, Riverside.
[e] Obtained from the culture collection of S. M. Mircetich, Department of Plant Pathology, University of California, Davis.
[f] Obtained from the culture collection of E. E. Butler, Department of Plant Pathology, University of California, Davis.
[g] Obtained from the culture collection of J. G. Hancock, Department of Plant Pathology, University of California, Berkeley.

supernatant was subjected to phenol extraction and then to chloroform-isoamyl alcohol (24:1 [vol/vol]) extraction before DNA was precipitated with ethanol. After centrifugation, pelleted DNA was resuspended in water and was precipitated again on ice for 1 h in 6.5% polyethylene glycol (PEG) 6,000 in the presence of 0.8 M NaCl (24). Pelleted DNA was washed in 70% ethanol and was dried under vacuum. The concentration and purity of the aqueous solution of DNA were determined spectrophotometrically.

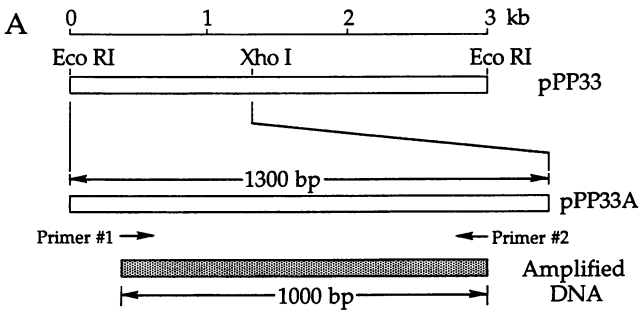
Recombinant DNA techniques. All plasmids were propagated in *Escherichia coli* JM101 (21). Methods for DNA restriction, ligation, transformation of the bacterium, and plasmid purification have been described previously (19). Restriction enzymes and T4 DNA ligase were purchased from Promega Corp. (Madison, Wis.). DNA was sequenced with oligonucleotide primers according to the dideoxy chain termination method of Sanger et al. (29). Enzymes and chemicals for DNA sequencing were purchased in the Sequenase Version 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio). Oligonucleotide primers for nucleotide sequencing were purchased from New England Biolabs (Beverly, Mass.).

Primer selection. Twenty-four-bp oligonucleotide primers were synthesized by the DNA Core Facility of the University of Missouri-Columbia. Oligonucleotide primers for the amplification of *P. parasitica* DNA were derived from pPP33, a plasmid that contains a 3.0-kb *P. parasitica*-specific DNA segment isolated from a library of *P. parasitica* isolate 5-3A (7, 9). To identify the primer sequences, a 1,300-bp *Eco*RI-*Xho*I DNA segment of pPP33 was subcloned into pUC18, which then was designated pPP33A. The nucleotide sequences of approximately 250 bp of DNA on both the 5′ and 3′ ends were determined. Oligonucleotide primers used for the amplification of *P. citrophthora* DNA were derived from pCIT15, a plasmid that contains a 5.1-kb *P. citrophthora*-specific DNA segment isolated from a library of *P. citrophthora* isolate P1323 (7, 10). To identify oligonucleotide primers for amplification of *P. citrophthora* DNA, an 800-bp *Pst*I DNA segment of pCIT15 (10) was subcloned into pUC18 and was designated pCIT15A. The nucleotide sequences of approximately 250 bp of DNA on both the 5′ and 3′ ends were determined.

PCR. A 100-μl reaction mixture was prepared which consisted of a commercial reaction buffer, 1.5 mM $MgCl_2$, 200 μM (unless otherwise stated) each dNTP, 2.5 U of *Taq* DNA polymerase (all purchased from Promega), and 100 pmol of each primer. Initially, 1 to 2 μg or 10-fold dilutions of *Phytophthora* spp. DNA from cultures or colonized plant tissue, respectively, or 100 ng of DNA from pPP33A and pCIT15A (positive controls) were added to the reaction mixtures. Reactions were cycled with an automated thermal cycler (Hybaid, model HB-TR1; National Labnet Co., Woodbridge, N.J.). The temperatures for denaturation and primer extension were 94 and 72°C, respectively (11, 20). The annealing temperatures for primers derived from *P. parasitica* and *P. citrophthora* were chosen empirically. The complete polymerization algorithm included 39 cycles of denaturation for 1 min at 94°C, annealing for 2 min at an appropriate temperature, and extension for 3 min at 72°C. These cycles were preceded by one cycle with an extended denaturation of 5 min and were concluded by one cycle with a final extension for 10 min at 72°C. Products from PCR (15 μl per lane) were electrophoresed on a 1% agarose gel with Tris-borate-EDTA buffer, stained with ethidium bromide, and then visualized with a UV transilluminator.

RESULTS

Investigation of PCR sensitivity. The 24-bp oligonucleotide primers for *P. parasitica* (Fig. 1B) and *P. citrophthora* (Fig. 2B) flanked the fungal DNA sequences of approximately 1,000 bp and 650 bp, respectively, that were to be amplified by PCR (Fig. 1A and 2A). Preliminary studies indicated that visible products could be obtained with approximately 30 thermocycles. Eventually, a 41-cycle algorithm was selected to provide



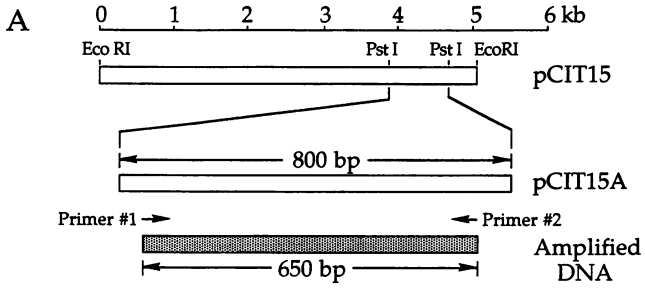
B Primer #1 5' - CTGACGATCCAGATCCTCTGCACG - 3'

Primer #2 5' - CTTGCGAGGCTTGACCGCTTCCTA - 3'

FIG. 1. (A) Derivation of the *P. parasitica*-specific primers from an *Eco*RI-*Xho*I subclone from the plasmid pPP33. (B) Oligonucleotide sequences of the *P. parasitica*-specific primers.

abundant amplification of species-specific sequences. The annealing temperature was a crucial factor in optimizing product formation, and the optimum temperature differed with the template-primer system. The primers for *P. parasitica* amplified DNA sequences of this species more specifically and efficiently at an annealing temperature of 65°C than at 60 or 70°C (Fig. 3). The most prominent of the amplified sequences of *P. parasitica* was a 1,000-bp product. This product migrated the same distance during electrophoresis as did the product of the plasmid pPP33A. When reactions were conducted at the optimal annealing temperature, as little as 10 to 20 pg of chromosomal DNA of *P. parasitica* was sufficient for detectable amplification of the specific DNA sequence (Fig. 4).

With *P. citrophthora*-specific primers, the highest yield of PCR products was obtained at an annealing temperature of 50°C; lower yields were obtained at 60°C (Fig. 5) or 45, 53, and 65°C (data not shown). One of the most prominent electrophoretic bands had an estimated size of 650 bp. This band matched that obtained from PCR with plasmid template pCIT15A (Fig. 5). At the annealing temperature of 50°C, 100 to 200 pg of chromosomal DNA of *P. citrophthora* was sufficient for detectable amplification of the specific DNA sequence (data not shown).



B Primer #1 5' - GTCGACGTCCTGCTTGGCACTCTG - 3'

Primer #2 5' - CGGTGCTCCGCGACTGTTGTCCAC - 3'

FIG. 2. (A) Derivation of the *P. citrophthora*-specific primers from a *Pst*I subclone from the plasmid pCIT15. (B) The oligonucleotide sequences of the *P. citrophthora*-specific primers.

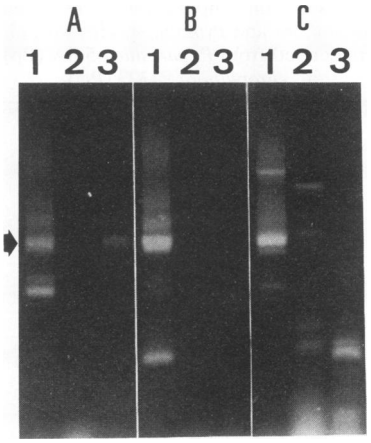


FIG. 3. Agarose gel pattern of PCR products amplified with *P. parasitica*-specific primers at annealing temperatures of 60°C (A), 65°C (B), and 70°C (C). Lanes 1, 2, and 3, chromosomal DNAs, 1 μg each, of *P. parasitica* 5-3A, *P. capsici* 1787, and *P. citrophthora* P1213, respectively. Arrow indicates the position of the 1,000-bp *P. parasitica*-specific product.

Specificity of PCR. Primers selected from *P. parasitica* 5-3A amplified the *P. parasitica*-specific DNA sequence from all the tested isolates of this species. Neither this sequence nor other sequences were ever amplified from any isolate of *P. citrophthora*, *P. capsici*, other *Phytophthora* spp., or *Pythium* spp. at the optimal annealing temperature of 65°C (Table 1). In addition to the 1,000-bp DNA sequence specific to *P. parasitica*, several nonspecific sequences also were amplified when large (1 to 2 μg) quantities of *P. parasitica* chromosomal DNA were added

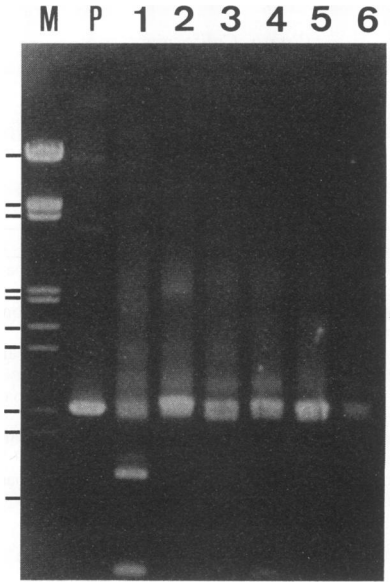


FIG. 4. Amplification of *P. parasitica* DNA sequences at 65°C annealing temperature with primers designed for this species, with various quantities of DNA to be amplified. Lanes 1 to 6, 2 μg, 200 ng, 20 ng, 2 ng, 200 pg, and 20 pg, respectively, of chromosomal DNA. Lane P, the 1,000-bp product amplified from 100 ng of plasmid pPP33A. Lane M, molecular size markers of lambda *Eco*RI-*Hin*dIII digest (Promega Corp.), with fragment sizes of 21,227, 5,148/4,973, 4,277, 2,027, 1,904, 1,584, 1,330, 983, 831, and 564 bp.

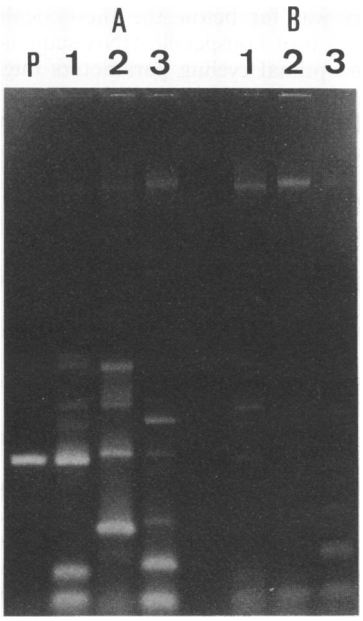


FIG. 5. PCR products generated with *P. citrophthora*-specific primers at annealing temperatures of 50°C (A) and 60°C (B). Lanes 1, 2, and 3, chromosomal DNAs (2 μg each) of *P. citrophthora* P1213, *P. capsici* 1787, and *P. parasitica* 5-3A, respectively. Lane P, the PCR product, ca. 650 bp, from 100 ng of pCIT15A.

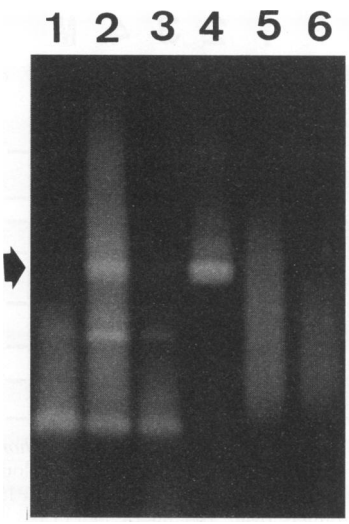


FIG. 7. Amplification of DNA from *P. parasitica*-infected or control tomato stems with *P. parasitica*-specific primers, as influenced by the amount of DNA added and the concentration of dNTPs, at 65°C annealing temperature. DNA was extracted 3 days after inoculation. Lanes 1 and 2, 5 μg and 1 μg of DNA from infected plant at dNTPs' concentration of 200 μM each. Lanes 3 and 4, 5 μg and 1 μg of DNA from infected plant at dNTPs' concentration of 50 μM each. Lanes 5 and 6, 5 μg and 1 μg of DNA from uninfected plant at dNTPs' concentration of 50 μM each. Arrow indicates the position of the 1,000-bp, *P. parasitica*-specific segment.

to the reaction mixture (Fig. 3 and 4). With reduction of the amount of DNA added, nonspecific bands could be eliminated on the agarose gel image (Fig. 4).

In addition to the expected 650-bp, *P. citrophthora*-specific sequence, species-nonspecific DNA sequences also were amplified with primers that were derived from *P. citrophthora* P1323 (Fig. 5). These primers also amplified DNA of *P. parasitica* and *P. capsici*, but not to the size of the *P. citrophthora*-specific sequence. Dilutions of the DNA preparations to the nanogram range eliminated nonspecific amplification by primers for *P. citrophthora* (Fig. 6). At these lower concentrations, only the 650-bp *P. citrophthora*-specific DNA sequence in all isolates of *P. citrophthora* tested was amplified. No amplification of this DNA sequence occurred with isolates of other randomly selected species of *Phytophthora* or *Pythium* (Table 1).

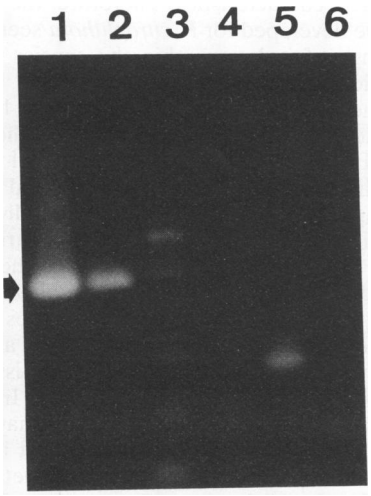


FIG. 6. PCR products amplified with *P. citrophthora*-specific primers as influenced by the amount of template DNA, at a 50°C annealing temperature. Lanes 1 and 2, *P. citrophthora* DNA; lanes 3 and 4, *P. parasitica* DNA; lanes 5 and 6, *P. capsici* DNA. Odd and even lane numbers correspond to DNA quantities of 1 μg and 10 ng, respectively. Arrow indicates the 650-bp, *P. citrophthora*-specific band.

Detection of *Phytophthora* spp. in tomato tissue. PCR also was applied to DNA extracted from tomato stem tissue adjacent to the site of wound inoculation with *P. parasitica*, *P. citrophthora*, or *P. capsici*. Primers derived from *P. parasitica* amplified the 1,000-bp species-specific sequence from DNA extracted from tomato 2 to 3 days after inoculation with this species (Fig. 7). The electrophoretic pattern of the PCR products was more ambiguous than the pattern of products from the chromosomal DNA extracted from pure cultures. The concentration of total (fungal and plant) DNA was crucial for electrophoretic resolution. For example, when 5 μg of total DNA was included in the reaction mixture, only a faint band with the size of the *P. parasitica*-specific segment (1,000 bp) was observed. This specific sequence was amplified more strongly when 1 μg of total DNA was included in the reaction mixture. Further improvement in specific amplification was achieved by decreasing the concentration of each dNTP from 200 μM to 50 μM. Under these conditions, the species-specific sequence was virtually the only reaction product (Fig. 7). Reduction of the quantity of total DNA to 100 ng did not produce detectable DNA at either concentration of dNTPs (data not shown).

Primers derived from *P. citrophthora* amplified DNA sequences only from tomato which had been inoculated with this species at least 6 to 7 days before DNA extraction. The 650-bp species-specific sequence was amplified most efficiently with 1 μg of total DNA at a concentration of 200 μM each dNTP

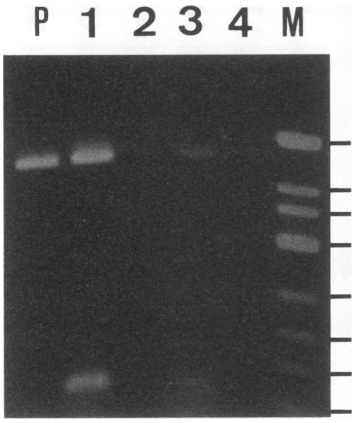


FIG. 8. Amplification of the 650-bp, *P. citrophthora*-specific DNA sequence from tomato stems inoculated 7 days previously with primers derived from the infectious species, *P. citrophthora* P1323. Lane P, the 650-bp band amplified from 100 ng of pCIT15A. Lanes 1 and 2, amplification products from 1 μg and 5 μg of total DNA, respectively, of infected plant tissue at dNTP concentrations of 200 μM each. Lanes 3 and 4, amplification from 1 μg and 5 μg of total DNA, respectively, at dNTP concentrations of 50 μM each. Lane M, X17 *Hin*fI marker (Promega Corp.) with fragments of 726/713, 553, 500, 427/413, 311, 299, 200, and 151 bp.

(Fig. 8). Neither set of primers amplified DNA from control plants or *P. capsici*-inoculated stem tissue.

DISCUSSION

Fungal pathogens in the genus *Phytophthora* are a threat to agriculturally important plants throughout the world. Plants can be infected simultaneously by two or more of these soil-borne fungal species. For example, solanaceous plants, such as tomato, can be infected by both *P. parasitica* and *P. capsici*, whereas citrus plants often are infected by *P. citrophthora* and *P. parasitica* at the same time. Little is known about how the development of one species of *Phytophthora* within infected plant tissue is influenced by the presence of another species. For example, do species which are non- or weakly pathogenic on a given plant interfere with those that are pathogenic on the same plant? It is also not clear whether populations of these parasites remain genetically distinct during the infection and colonization processes. The assessment of in planta events is hampered by morphological similarities among species (3, 25) and by the lack of reliable genetic markers.

Genetic markers for the present study were elaborated on the basis of the presence of species-specific sequences in the chromosomal DNA of *P. parasitica* and *P. citrophthora* (7, 9, 10). Sufficiently stringent PCR conditions were established to differentiate these two species from each other and from other *Phytophthora* spp. in both aseptic cultures and infected plants.

Oligonucleotide primers were selected which would amplify 1.0- and 0.65-kb regions of species-specific DNA of *P. parasitica* and *P. citrophthora*, respectively. Fragments of DNA in these size classes fell within the range suggested to be optimal for amplification by PCR (11, 20, 22).

The rate and specificity of amplification were influenced strongly by annealing temperature. The theoretical annealing temperature (11) calculated from primers derived from *P. parasitica* was 71°C. This value was slightly higher than the empirically derived optimal temperature of 65°C. The optimal annealing temperature of 50°C selected for *P. citrophthora*-specific primers was far below the theoretical optimum of 74°C. Amplification of nonspecific DNA sequences at suboptimal and even optimal cycling parameters might be due to mispriming and/or misextension that occurs with increasing amounts of DNA and/or dNTPs. Indeed, reduction of DNA quantities added to the reaction mixture led to the elimination of unexpected amplification products. As little as 10 to 20 pg of *P. parasitica* DNA and 100 to 200 pg of *P. citrophthora* DNA were sufficient for detectable amplification of the respective species-specific sequences. In addition to nonspecific products, insufficient amplification of target sequences also occurred with increasing quantities of total, and obviously contaminated, DNA from infected plant tissue as the source of template DNA.

Goodwin et al. (7, 8, 9, 10) established an unambiguous method for identification of *Phytophthora* spp. by means of labeled species-specific DNA probes. With probes such as pPP33 and pCIT15, as little as 1 ng of DNA from *P. parasitica* and *P. citrophthora*, respectively, was identifiable (9, 10). Our PCR method was capable of detecting at least 1 order of magnitude less DNA, more quickly and without radioisotopes. The higher sensitivity of the PCR approach may be due both to the technique per se and to size differences of DNA fragments used as markers.

The repetitive nature of probes pPP33 and pCIT15 was demonstrated by their abilities to hybridize with numerous fragments of digested chromosomal DNA (9, 10). Using methods identical to those of Goodwin et al. (9, 10), we also observed hybridization of the smaller 1,000- and 650-bp DNA fragments to numerous fragments of digested DNA of *P. parasitica* and *P. citrophthora*, respectively (5a).

Our primers appear to be specific for DNAs from a number of isolates of the two species. A 1,000-bp segment specific to *P. parasitica* was amplified only from DNAs of isolates of this species from a variety of hosts and geographic sources. The *P. citrophthora*-specific, 650-bp sequence was amplified only from DNAs of isolates of *P. citrophthora*, including P1213, P1201, and P449 from cacao in Brazil (Table 1). This latter observation is of interest because the pCIT15 probe did not hybridize with DNA from these isolates in earlier studies (10). All of these isolates had been identified as *P. citrophthora* on the basis of morphological characteristics. Therefore, the PCR marker system we have developed for *P. citrophthora* seems to identify a broader range of isolates within the species than did the previous hybridization system.

Although morphology is likely to continue to be relevant in *Phytophthora* identification, the involvement of molecular techniques should help overcome ambiguities and uncertainties occurring in this field. The use of morphological criteria often fails, for example, when variability within an individual species occurs; fungicide action is a frequent cause of variability (3). In this regard, PCR has allowed us to detect the 1,000-bp *P. parasitica*-specific and the 650-bp *P. citrophthora*-specific segments in drug-resistant mutants of these species (5a, 31).

P. citrophthora has not been referred to as a pathogen of tomato, yet it was able to slowly colonize stem tissues of young tomato plants following wound inoculation. Infection of a plant by this type of weakly virulent pathogen may occur under natural conditions and yet be difficult to detect if populations of the pathogen in plant tissue remain low. Detection of low populations of such a pathogen and evaluation of the potential for these populations to interact genetically with cocolonizing virulent pathogens require a sensitive means of distinguishing among the pathogen species. The development of genetic markers for *P. parasitica* and *P. citrophthora*, based on PCR methods, provides a means of beginning such genetic and

population studies in this model host-parasite system. Application of PCR technology to routine detection of *Phytophthora* spp. in symptomatic plant material will require further optimization of reaction conditions and, perhaps, further screening of potential primer sequences.

ACKNOWLEDGMENTS

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