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PCR Amplification of Ribosomal DNA for Species Identification in the Plant Pathogen Genus *Phytophthora*

JEAN B. RISTAINO,* MICHAEL MADRITCH, CAROL L. TROUT, AND GREGORY PARRA

Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina 27695

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We have developed a PCR procedure to amplify DNA for quick identification of the economically important species from each of the six taxonomic groups in the plant pathogen genus *Phytophthora*. This procedure involves amplification of the 5.8S ribosomal DNA gene and internal transcribed spacers (ITS) with the ITS primers ITS 5 and ITS 4. Restriction digests of the amplified DNA products were conducted with the restriction enzymes *Rsa*I, *Msp*I, and *Hae*III. Restriction fragment patterns were similar after digestions with *Rsa*I for the following species: *P. capsici* and *P. citricola*; *P. infestans*, *P. cactorum*, and *P. mirabilis*; *P. fragariae*, *P. cinnamomi*, and *P. megasperma* from peach; *P. palmivora*, *P. citrophthora*, *P. erythroseptica*, and *P. cryptogea*; and *P. megasperma* from raspberry and *P. sojae*. Restriction digests with *Msp*I separated *P. capsici* from *P. citricola* and separated *P. cactorum* from *P. infestans* and *P. mirabilis*. Restriction digests with *Hae*III separated *P. citrophthora* from *P. cryptogea*, *P. cinnamomi* from *P. fragariae* and *P. megasperma* on peach, *P. palmivora* from *P. citrophthora*, and *P. megasperma* on raspberry from *P. sojae*. *P. infestans* and *P. mirabilis* digests were identical and *P. cryptogea* and *P. erythroseptica* digests were identical with all restriction enzymes tested. A unique DNA sequence from the ITS region I in *P. capsici* was used to develop a primer called PCAP. The PCAP primer was used in PCRs with ITS 1 and amplified only isolates of *P. capsici*, *P. citricola*, and *P. citrophthora* and not 13 other species in the genus. Restriction digests with *Msp*I separated *P. capsici* from the other two species. PCR was superior to traditional isolation methods for detection of *P. capsici* in infected bell pepper tissue in field samples. The techniques described will provide a powerful tool for identification of the major species in the genus *Phytophthora*.

Phytophthora species are responsible for economically important diseases of a wide range of agronomic and ornamental crops. Species identification for *Phytophthora* has traditionally been based upon microscopic examination of morphological characters and growth characteristics of the pathogen on specific media (27, 35). Variations in the morphological characters of both the sexual and asexual stages of this group of pathogens exist, leading to difficulties in accurate identification by traditional methods. In addition, identification based on pathogenicity assays or growth characteristics are time-consuming. Accurate and rapid identification of *Phytophthora* species in plant material is important for several reasons. First, in many hosts, such as citrus, walnut, strawberry, raspberry, potato, and tomato, multiple species of *Phytophthora* can infect the plant, and the relative severity of disease and the plant part infected can vary among pathogen species (6, 25, 37, 43). Preplant identification of *Phytophthora* species can be important for quarantine purposes and is important for restricting the spread of pathogens in plant material (23). In addition, accurate diagnosis of the species of *Phytophthora* is important in disease management and control.

Molecular tools including isozyme analysis, restriction fragment length polymorphisms in nuclear and mitochondrial DNA, randomly amplified polymorphic DNA PCRs, serological assays, DNA probes, and PCR of internal transcribed spacer (ITS) regions and nuclear small- and large-subunit ribosomal DNA (rDNA) have been used to evaluate intraspecific and interspecific variation in *Phytophthora* species (1, 5, 8, 11, 13, 14, 22, 28). Molecular techniques have also been used to study genetic diversity and evolutionary origins in populations of many different fungal genera (2). Nucleotide sequences of rRNA genes have been used in studies of phylogenetic relationships over a wide range of taxonomic levels with many organisms (2, 9, 31, 41). The nuclear small-subunit rDNA sequences evolve relatively slowly and are useful for studying distantly related organisms, whereas the ITS regions and intergenic region of the nuclear rRNA repeat units evolve the fastest and may vary among species and populations (41). Mitochondrial rRNA genes also evolve rapidly and can be useful at the ordinal or family level (41). The evolutionary lineage of the oomycetes has been elucidated by sequencing studies with small-subunit rRNA sequences (9).

We have adopted a quick extraction procedure for DNA and a reliable PCR technique for amplification of DNA from *Phytophthora* species. This method is based on procedures developed by Lee and Taylor (21) and Lee et al. (22) for *Phytophthora* species and involves amplification of the ITS and 5.8S rDNA. We used ITS primers 5 and 4 and PCR to amplify the entire 5.8S rDNA gene, both ITS regions I and II, and a portion of the 18S nuclear small-subunit rDNA gene. The amplified DNA was then cut with a series of restriction enzymes to develop species-specific restriction fragment patterns for rapid identification of the important plant-pathogenic *Phytophthora* species from all the different taxonomic groups in the genus that infect economically important hosts (35). In addition, we devised a PCR primer to specifically amplify *P. capsici*, an important pathogen of pepper, and used this primer (PCAP) to compare PCR to traditional isolation methods for identification of the pathogen in infected pepper tissue from the field.

MATERIALS AND METHODS

Culture preparation and PCR methods. Mycelium of each *Phytophthora* species was grown in pea broth. Pea broth was prepared by autoclaving 120 g of

* Corresponding author. Mailing address: Box 7616, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695. Phone: (919) 515-3257. Fax: (919) 515-7716. E-mail: Jean_Ristaino@ncsu.edu.

frozen peas in 500 ml of distilled water for 5 min. The filtrate was brought to 1 liter with distilled water and autoclaved for 25 min. Multiple cultures of authenticated isolates from each of the six taxonomic groups in the genus, including group I, *P. cactorum* (Lebert & Cohn) Schroter; group II, *P. capsici* Leonian, *P. citrophthora* (R. E. Sm & E. H. Smith), *P. nicotianae* Breda de Haan (15), *P. palmivora* (E. Butler); group III, *P. citricola* (Saw.); group IV, *P. infestans* (Mont.) de Bary and *P. mirabilis*; group V, *P. fragariae* (C. J. Hickman), *P. megasperma* (Drechs.), and *P. sojae* (Hildebr.); and group VI, *P. cinnamomi* (Rands), *P. cryptogea* (Pethybr. & Laff.), and *P. erythroseptica* (Pethybr.), were collected from researchers (Table 1). These taxonomic groups are based on growth characteristics of the pathogen on media, morphological characters of the sexual and asexual propagules, and cardinal temperatures for growth (35). Isolates of *P. infestans* and *P. fragariae* were grown in pea broth for 1 week at 18 and 20°C, respectively, while the other species were grown in pea broth for 1 week at 25°C. Mycelium was filtered from the pea broth and frozen in cryogenic vials at −20°C for subsequent work.

DNA was extracted from frozen mycelium by a CTAB (hexadecyltrimethylammonium bromide) procedure (19). Frozen mycelium was placed in 1.5-ml microtubes, 150 μl of extraction buffer (0.35 M sorbitol, 0.1 M Tris, 0.005 M EDTA [pH 7.5], 0.02 M sodium bisulfite) was added, and the tubes were vortexed. Nuclear lysis buffer (150 μl) containing 0.2 M Tris, 0.05 M EDTA (pH 7.5), 2.0 M NaCl, and 2% CTAB (pH 7.5) was added, followed by 60 μl of 5% Sarkosyl (5 g *N*-lauroylsarcosine per 100 ml of H_2O), and the tubes were vortexed and then incubated at 65°C for 15 to 30 min. Chloroform-isoamyl alcohol (24:1 mixture of chloroform and isoamyl alcohol) (1 volume) was added to each tube, and the tubes were mixed and centrifuged for 15 min at 13,000 × *g*. The aqueous phase was transferred to a new tube, and the chloroform extraction was repeated. DNA was precipitated overnight at −20°C after the addition of 0.1 volume of 3 M sodium acetate (pH 8.0) and 2 volumes of cold 100% ethanol. The supernatant was discarded, and the pellets were washed with 70% ethanol and then dried by vacuum centrifugation. DNA was resuspended in 100 μl of TE (10 mM Tris-HCl, 0.1 mM EDTA [pH 8.0]) and then diluted 1:100 for use in PCRs in TE. Extracted DNA was electrophoresed in 1% agarose gels at 25 mA for 3 h. The gels were stained for 15 min in ethidium bromide (0.5 μg/ml) and destained for 15 min in distilled water; alternatively, ethidium bromide was incorporated directly into the gels at a rate of 0.5 μg/ml. The gels were photographed under UV light, and digital images were scanned onto diskettes with a gel scanner (UVP Imagestore 7500).

PCRs were conducted in 50-μl reaction volumes. Each reaction tube contained approximately 1 μl of a 1-ng/μl DNA template, 5 μl of 10× PCR buffer (Boehringer Mannheim, Indianapolis, Ind.), 36.6 μl of sterile distilled water, 2 μl (each) of 1.25 mM deoxynucleoside triphosphates (Pharmacia Biotech, Piscataway, N.J.), 2 μl of 10 mM $MgCl_2$ (Sigma, St. Louis, Mo.), 2 μl each of 10 μM forward and reverse primers (41), and 0.4 μl of *Taq* (5 U/μl; Boehringer Mannheim). Two drops of mineral oil was placed on the top of each reaction mixture before thermal cycling. The thermal cycling parameters were initial denaturation at 96°C for 2 min followed by 35 cycles consisting of denaturation at 96°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension at 72°C for 10 min was done at the end of the amplification. Negative controls (no DNA template) were used in every experiment to test for the presence of contamination in reagents. Separate pipettes fitted with filter pipette tips were used in a UV-irradiated hood to prepare master mix reagents for PCR. DNA was pipetted in a separate location with different pipettes.

The ITS primers ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG) and ITS 4 (5′-TCCTCCGCTTATTGATATGC) (41) amplify the ITS region I between the 18S and 5.8S rDNAs, the 5.8S rDNA, the ITS region II, and a portion of the 28S rDNA. In the first experiments with *P. infestans*, we used the three primer pairs ITS 5 and ITS 4, ITS 5 and ITS 2 (5′ GCTGCGTTCTTCATCGATGC), and ITS 3 (5′-GCATCGATGAAGAACGCAGC) and ITS 4. All the primer sequences are written 5′ to 3′. Odd-numbered primers are 5′-to-3′ primers, and even-numbered primers are 3′-to-5′ primers. ITS region I between the 18S rDNA and the 5.8S rDNA is flanked by ITS 5 and ITS 2 (41). ITS region II between the 5.8S rDNA and the 28S rDNA is flanked by ITS 3 and ITS 4 (41). In subsequent experiments, primers ITS 4 and ITS 5 were used with all the *Phytophthora* species.

Amplified fragments were digested with the restriction enzymes *Rsa*I, *Hae*III, and/or *Msp*I. Restriction digests consisted of 3 μl of enzyme mixture (1 μl of REact buffer [Gibco BRL, Gaithersburg, Md.], 1 μl of restriction enzyme, and 8 μl of sterile distilled water) and 30 μl of amplified PCR product. DNA was digested at 37°C for 1.5 h and then at 65°C for 10 min. Digested DNA was electrophoresed on a 2% agarose gel at 25 mA for 3 h. The gels were stained in ethidium bromide (0.5 μg/ml) to visualize polymorphisms in amplified DNA fragments. The sizes of the restriction fragments of all the species were measured directly from the same gels and compared to standards ladders. Fragment sizes in base pairs were calculated with the shareware program SEQAID II (32). Representative restriction fragment patterns of individual isolates are shown in the figures; however, all the isolates in Table 1 were tested in individual experiments.

Development of a *P. capsici*-specific primer. DNA from two isolates of *P. capsici* (B1HB14 and B2HH4) was amplified with PCR primers ITS 1 (5′-TCCGT AGGTGAACCTGCGG) and ITS 4. The amplified DNA was cleaned with a Gene Clean kit (Bio 101, Vista, Calif.) by standard procedures. DNA from the two isolates was subjected to automated DNA sequencing on a Perkin-Elmer DNA sequencer at the Iowa State University DNA Sequencing Facility (Ames, Iowa). The DNA sequences were aligned with published sequences from five other *Phytophthora* species (21) by using the sequence alignment program CLUSTAL (18). Regions of dissimilarity in the ITS region I were used to design and construct a primer specific for *P. capsici*, called the PCAP primer. The best sequence for the PCAP primer was 5′-TAATCAGTTTTGTGAAATGG. This sequence was published by Lee and Taylor (22) and was developed as an oligonucleotide probe for *P. capsici*. The PCAP primer was paired with primer ITS 1 and tested with 38 isolates of *P. capsici* obtained from a variety of vegetable hosts including pepper, tomato, pumpkin, squash, and cucumber (Table 1). The PCAP primer was also tested on isolates comprising 13 different species of *Phytophthora* (Table 1) in PCRs as described above.

PCR detection in plant tissue. Field samples of bell pepper that either were asymptomatic, contained visible lesions, or were dead from infections caused by *P. capsici* were sampled from field plots in 1995. The lesions were cut in half to compare recovery after culture on isolation media to the PCR method. The tissue was surface disinfested in 0.05% sodium hypochlorite and plated on a semiselective medium for isolation of the pathogen (20). For PCR, a portion of the remaining lesion (10 mg) was lysed with 0.5 N NaOH (10 μl/mg), and then 5 μl was diluted immediately in 495 μl of 100 mM Tris buffer (pH 8.0) (39). A 1-μl volume of this extract was used as the DNA template for PCR with the PCAP and ITS 1 primers. Twenty-five plants from each symptom category were sampled, and the PCR experiments were repeated twice.

Nucleotide sequence accession numbers. The complete ITS sequences of the two pepper isolates of *P. capsici* have been submitted to the GenBank at the National Center for Biotechnology Information (accession no. AF007021 and AF007022).

RESULTS

DNA extracted from *P. infestans* was amplified with ITS primer pairs ITS 5/2, ITS 5/4, and ITS 3/4 (Fig. 1). *Pythium ultimum*, a related oomycete in a different genus, was amplified for comparison (Fig. 1, lanes 4, 8, and 12). PCR amplification of *P. infestans* with ITS primers 5/2, 5/4, and 3/4 yielded an estimated 363-bp product, a 946-bp product, and a 612-bp product, respectively. PCR amplification of *P. ultimum* with ITS primers 5/4 and 3/4 yielded slightly larger products than did amplification of *P. infestans*, whereas amplification of *P. ultimum* with ITS 5/2 yielded a similar-size product (Fig. 1).

P. infestans ITS DNA was digested with a panel of restriction enzymes. Restriction analysis of ITS DNA and 5.8S rDNA from *P. infestans* amplified with primer pairs ITS 5/2, ITS 5/4, and ITS 3/4 was conducted. Restriction digests with *Bst*NI, *Hha*I, *Hin*fI, *Rsa*I, *Pst*I, and *Hae*III were done. None of the enzymes tested digested the 363-bp product from *P. infestans*, but *Rsa*I cut the larger 946-bp product into smaller fragments approximately 433, 286, 100, and 79 bp in length. Restriction sites for enzymes *Bst*NI, *Hha*I, and *Hin*fI were also found in the amplified 946-bp fragment and the 612-bp fragment.

Amplified rDNA from isolates of *P. cactorum* from taxonomic group I was digested with *Rsa*I, *Msp*I, and *Hae*III. Four restriction fragments were observed in *P. cactorum* after digestion with *Rsa*I (Fig. 2, lane 2; Table 2). The restriction fragment pattern for *P. cactorum* was identical to the patterns observed for *P. infestans* and *P. mirabilis* (Fig. 3, lanes 2 to 4). *Msp*I digests of amplified DNA distinguished *P. cactorum* (Fig. 4, lane 2; Table 3) from *P. infestans* and *P. mirabilis* (Fig. 4, lanes 3 and 4; Table 3). *P. infestans* and *P. mirabilis* had identical restriction fragment patterns when digested with *Msp*I (Fig. 4, lanes 3 and 4). Restriction sites for *Hae*III were not found in amplified DNA from *P. infestans* and *P. mirabilis*, but two fragments of approximately 717 and 189 bp were observed in digested rDNA of *P. cactorum* (Table 3).

Restriction fragment patterns were similar between all taxonomic group II isolates of *P. capsici* tested (Fig. 2, lane 3; Table 1) and taxonomic group III isolates of *P. citricola* (Fig. 2, lane 10). Since *P. capsici* and *P. citricola* had similar restriction fragment patterns in this amplified region of DNA, further digests with other restriction enzymes were done. Isolates of

TABLE 1. Species, plant host, source and designation of isolates of *Phytophthora* species used in PCR experiments

Species and taxonomic group[a]	Plant host (no. of isolates)	Isolate designation and source[b]
Group I		
P. cactorum	Unknown (3)	127 77, 234 81 (L. Cooke); 1298 (G. Weidemann)
Group II		
P. capsici	Pepper (24)	1, 17–33, 82–88 (J. Ristaino)
	Tomato (5)	34–38 (J. Ristaino)
	Pumpkin (3)	39–41 (J. Ristaino)
	Squash (3)	52, 55, 57 (J. Ristaino)
	Cucumber (1)	61 (J. Ristaino)
P. citrophthora	Citrus (5)	M86, M139, M140, M189, M259 (J. Menge)
	Walnut (1)	34-4-7 (J. Mircetich)
P. nicotianae	Tobacco (5)	Rmt 6, 332, 340, 335, 435 (D. Shew)
	Tomato (5)	1-3A, 6-1A, 5-3A, 2HB, 6-H (J. Ristaino)
	Walnut (1)	35-1-5 (J. Mircetich)
	Boxwood (1)	2107 (M. Benson)
	Vinca (1)	2127 (M. Benson)
	Rhododendron (1)	2109, 116 (M. Benson)
	Azalea (1)	2121 (M. Benson)
	Citrus (5)	D-1, R-1, H-2, BHG-1, B-1 (J. Graham)
P. palmivora	Milkweed (1)	P66 (J. Graham)
	Citrus (6)	P8, P29, P40, P44, P48, Shaw (J. Graham)
Group III		
P. citricola	Avocado (5)	M213, M215, M220, M265, M266 (J. Menge)
Group IV		
P. infestans	Tomato (5)	US-7 NY (W. Fry); PINC 93-2, PINC 93-1, PINC 93-4, PINC 93-5 (P. Shoemaker)
	Potato (8)	US-6 NY (W. Fry); PINC 94-8-1, PINC 94-19, PINC 94-1, PINC 94-7, PINC 94-37 (P. Shoemaker); US-1, US-8 (B. Christ)
P. mirabilis	*Mirabilis jalapa* (1)	0S0016 (W. Fry)
Group V		
P. fragariae	Strawberry (5)	A-8, R-4, NC-1, R-1, R-6 (B. Milholland)
P. megasperma	Raspberry (2)	NY 318, NY 321 (W. Wilcox)
	Apricot (1)	NY 222 (W. Wilcox)
	Cherry (2)	NY 344, NY 346 (W. Wilcox)
	Peach (1)	NY 412 (W. Wilcox)
	Walnut (1)	33-2-9 (J. Mircetich)
P. sojae	Soybean (6)	R1, R3, R4, R8, R13, R25 (X. Yang)
Group VI		
P. cinnamomi	Rhododendron (1)	2301 (M. Benson)
	Fraser fir (1)	2302 (M. Benson)
	Camellia (1)	2322 (M. Benson)
	Shore juniper (1)	2325 (M. Benson)
	Leucothe (1)	2349 (M. Benson)
	Walnut (1)	34-2-8 (J. Mircetich)
P. cryptogea	Safflower (2)	PCR-1, 34-1-7 (J. Duniway)
P. erythroseptica	Potato (3)	4, 10, 1 (J. Duniway)

[a] Groups I to VI are taxonomic groups devised by Waterhouse (40) and Stamps et al. (35) to separate the species within the genus. Taxonomic groups are based on morphological and physiological characters of the pathogen.

[b] Isolate designation and source indicates the isolate number and name of the investigator from whom the culture was obtained.

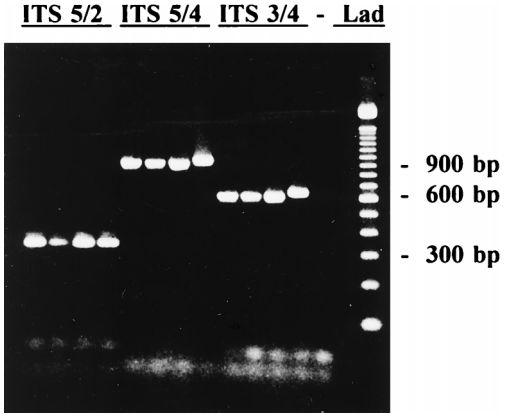

FIG. 1. Extracted DNA of *P. infestans* was amplified with primer pairs ITS 5 and 2, ITS 5 and 4, and ITS 3 and 4. *Pythium ultimum* DNA amplified with the same primer pairs is shown in the intervening lanes. The no-template control (−) and 100-bp DNA ladder (Lad) are also shown.

P. capsici were differentiated from isolates of *P. citricola* after digestion with *Msp*I (Fig. 4, lanes 5 and 6; Table 3). In contrast, taxonomic group II isolates of *P. citrophthora* from citrus (Fig. 2, lane 4; Table 2) had different restriction fragment length patterns from *P. capsici* and *P. citricola* after digestion with *Rsa*I (Fig. 2, lanes 3 and 10). Only one isolate of *P. citrophthora* from walnut was tested in our study (lane 5), and it had a slightly different restriction fragment pattern from the citrus isolates of *P. citrophthora* (lane 4). Other isolates from walnut need to be tested to confirm or refute this restriction fragment pattern for the walnut *P. citrophthora.*

Isolates of *P. nicotianae* (formerly *P. parasitica*) from tobacco, tomato, walnut, boxwood, vinca, rhododendron, azalea, and citrus are classified into taxonomic group II (Table 1). All isolates of *P. nicotianae* tested had the same restriction fragment patterns in this amplified region of ITS and 5.8S rDNA, and four fragments were visible after restriction digestion with *Rsa*I (Fig. 2, lanes 6 to 8; Tables 1 and 2).

P. palmivora from citrus (Fig. 2, lane 9) had a similar restriction fragment pattern to *P. citrophthora* from citrus (lane 4) when digested with *Rsa*I but had a different pattern from isolates of *P. nicotianae* from citrus (lane 6). *P. palmivora* could be

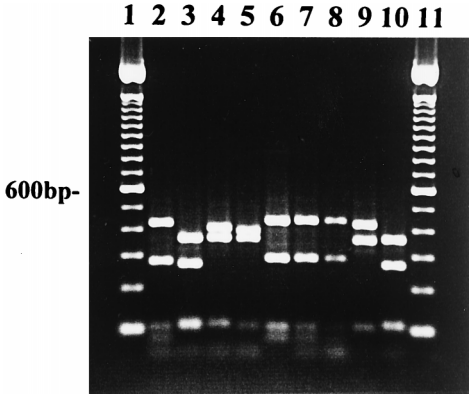

FIG. 2. Restriction analysis with *Rsa*I of DNA amplified with primer pair ITS 5 and ITS 4 from *P. cactorum* 1298 (lane 2), *P. capsici* B1HB14 (lane 3), *P. citrophthora* M86 (lane 4), *P. citrophthora* 34-4-7 (lane 5), *P. nicotianae* D-1 (lane 6), *P. nicotianae* Rmt 6 (lane 7), *P. nicotianae* 1-3A (lane 8), *P. palmivora* P8 (lane 9), and *P. citricola* M213 (lane 10). Lanes 1 and 11 contain 100-bp ladders.

TABLE 2. Restriction fragment sizes from ITS and 5.8S rDNA of *Phytophthora* species amplified with ITS primers 5 and 4[a]

Species and taxonomic group[b]	Fragment sizes (bp)	Nondigested-product size (bp)
Group I		
*P. cactorum***	436, 286, 111, 79	925
Group II		
*P. capsici**	369, 278, 116	899
P. citrophthora from citrus****	426, 378, 122	925
P. citrophthora from walnut	416, 378, 122	925
P. nicotianae	456, 310, 116, 92	925–980
*P. palmivora****	436, 369, 116	952
Group III		
*P. citricola**	378, 286, 122	925
Group IV		
*P. infestans***	433, 286, 100, 79	925
*P. mirabilis***	433, 286, 100, 79	925
Group V		
*P. fragariae****	454, 225, 178, 111	980
P. megasperma from raspberry*****	454, 393, 111	980
P. megasperma from peach***	454, 218, 184, 111	980
*P. sojae*****	454, 393, 111	980
Group VI		
*P. cinnamomi****	454, 218, 184, 111	980
*P. cryptogea*****	423, 383, 111	925
*P. erythroseptica*****	423, 383, 111	925

[a] Amplified DNA was digested with *Rsa*I.
[b] Species with the same number of asterisks have similar restriction fragment patterns after digestions with *Rsa*I.

distinguished from *P. citrophthora* after digestion with *Hae*III (Fig. 5, lanes 2 and 5; Table 3). *P. palmivora* was not digested by *Hae*III, but *P. citrophthora* was digested (Fig. 5, lanes 2 and 5; Table 3).

All taxonomic group IV isolates of *P. infestans* from potato and tomato showed the same restriction fragment pattern after digestion with *Rsa*I (Fig. 3, lanes 2 and 3). Four fragments were

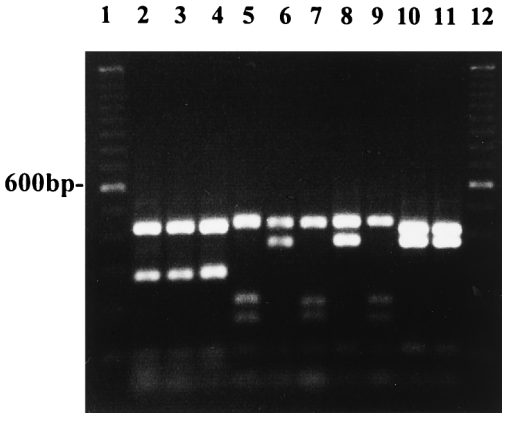

FIG. 3. Restriction analysis with *Rsa*I of DNA amplified with primer pair ITS 5 and ITS 4 from *P. infestans* US-6 NY (lane 2), *P. infestans* 93-1 (lane 3), *P. mirabilis* 0S0016 (lane 4), *P. fragariae* A-8 (lane 5), *P. megasperma* NY 318 (lane 6), *P. megasperma* NY 412 (lane 7), *P. sojae* R1 (lane 8), *P. cinnamomi* 2302 (lane 9), *P. cryptogea* PCR-1 (lane 10), and *P. erythroseptica* 4 (lane 11). Lanes 1 and 12 contain 100-bp ladders.

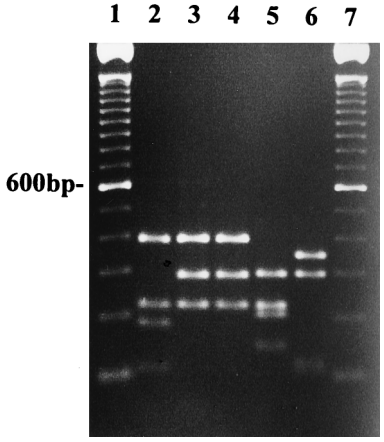

FIG. 4. Restriction analysis with *Msp*I of DNA amplified with ITS 5 and ITS 4 from *P. cactorum* 1298 (lane 2), *P. infestans* US-6 NY (lane 3), *P. mirabilis* 0S0016 (lane 4), *P. capsici* B1HB14 (lane 5), and *P. citricola* M213 (lane 6). Lanes 1 and 7 contain 100-bp ladders.

observed, and the restriction patterns were identical to those of *P. mirabilis* (Fig. 3, lane 4; Table 2). In contrast, *P. erythroseptica*, which causes pink rot of potato, gave a restriction fragment pattern different from that of *P. infestans* in this amplified region when digested with *Rsa*I (Fig. 3, lane 11; Table 2).

All isolates of *P. fragariae* (taxonomic group V) from strawberry had identical restriction fragment patterns when amplified DNA was digested with *Rsa*I and yielded four fragments (Fig. 3, lane 5). Variation in the restriction fragment patterns was observed within the group of isolates identified as *P. megasperma*. Isolates of *P. megasperma* from raspberry (Fig. 3, lane 6), apricot, and cherry had the same restriction fragment patterns when digested with *Rsa*I (Tables 1 and 2). However, the putative isolates of *P. megasperma* from peach (Fig. 3, lane 7) and walnut (not shown) had restriction fragment patterns similar to *P. fragariae* (lane 5) and *P. cinnamomi* (lane 9) after digestion with *Rsa*I. *P. sojae* isolates from soybean (lane 8) had identical restriction fragment patterns to *P. megasperma* from

TABLE 3. Restriction fragment sizes from ITS and 5.8S rDNA of *Phytophthora* species amplified with ITS primers 5 and 4[a]

Species	Fragment sizes (bp)
Digestion with *Msp*I	
P. capsici	290, 219, 194, 140
P. citricola	340, 274, 111
P. cactorum	396, 225, 189, 109
P. infestans	397, 293, 225
P. mirabilis	397, 293, 225
Digestion with *Hae*III	
P. cactorum	717, 189
P. palmivora	920
P. erythroseptica	749, 122
P. cryptogea	749, 122
P. citrophthora	481, 306
P. cinnamomi	446, 319
P. fragariae	347, 179, 145, 106
P. megasperma from peach	463, 315, 128
P. sojae	402, 315, 101
P. megasperma from raspberry	475, 302, 108

[a] Amplified DNA was digested with either *Msp*I or *Hae*III.

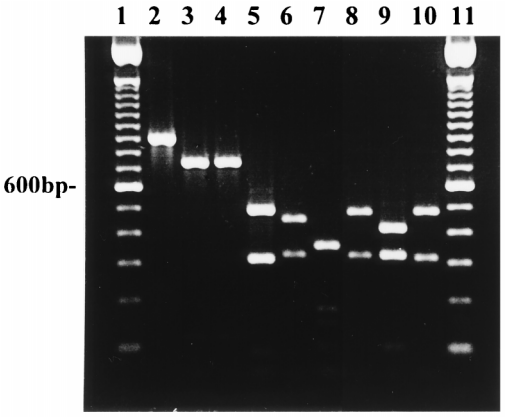

FIG. 5. Restriction analysis with *Hae*III of DNA amplified with ITS 5 and ITS 4 from *P. palmivora* P8 (lane 2), *P. erythroseptica* 4 (lane 3), *P. cryptogea* PCR-1 (lane 4), *P. citrophthora* M86 (lane 5), *P. cinnamomi* 2302 (lane 6), *P. fragariae* A-8 (lane 7), *P. megasperma* NY 412 (lane 8), *P. sojae* R1 (lane 9), and *P. megasperma* NY 318 (lane 10). Lanes 1 and 11 contain 100-bp ladders.

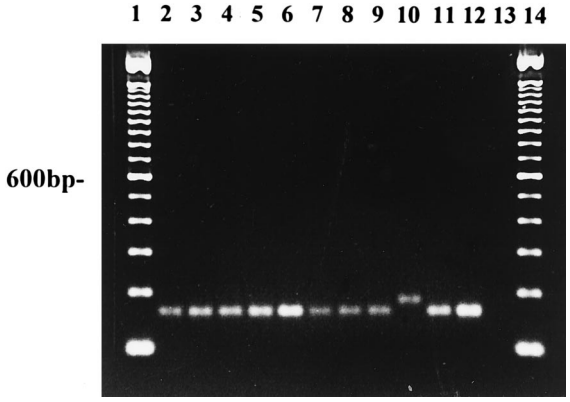

FIG. 6. DNA amplified with the PCAP primer and ITS 1 from *P. capsici* 17 (lane 2), *P. capsici* 18 (lane 3), *P. capsici* 19 (lane 4), *P. capsici* 20 (lane 5), *P. capsici* 21 (lane 6), *P. capsici* 22 (lane 7), *P. capsici* 23 (lane 8), *P. capsici* 25 (lane 9), *P. citrophthora* M86 (lane 10), *P. citricola* M213 (lane 11), and *P. capsici* 87 (lane 12). Lanes 1 and 14 contain 100-bp ladders; lane 13 contains a no-template control.

raspberry, apricot, and cherry when digested with *Rsa*I (Fig. 3, lane 6; Table 2). However, restriction digestion with *Hae*III separated these two species (Fig. 5, lanes 8 and 9; Table 3).

All the isolates of *P. cinnamomi* (taxonomic group VI) from a variety of hosts including rhododendron, fraser fir, camellia, shore juniper, and leucothe (Table 1) had similar restriction fragment patterns when digested with *Rsa*I and yielded four fragments (Fig. 3, lane 9; Table 2). These bands were similar in size to the restriction fragments observed when DNA from *P. fragariae* and *P. megasperma* from peach were digested with *Rsa*I (Fig. 3, lanes 5 and 7; Table 2). Digestion of amplified DNA with *Hae*III differentiated *P. cinnamomi* from *P. fragariae* (Fig. 5, lanes 6 and 7). Both *P. cryptogea* isolates from safflower had the same restriction fragment patterns when digested with *Rsa*I and yielded three fragments (Fig. 3, lanes 10). These restriction fragments were similar to those of *Rsa*I-digested *P. citrophthora* (Fig. 2, lane 4) and *P. erythroseptica* (Fig. 3, lane 11). However, digestion of amplified DNA with *Hae*III differentiated isolates of *P. cryptogea* and *P. erythroseptica* (Fig. 5, lanes 3 and 4; Table 3) from *P. citrophthora* (Fig. 5, lane 5). *P. erythroseptica* and *P. cryptogea* had the same restriction fragment patterns after digestion with *Hae*III, and we were unable to distinguish between these two species with the range of restriction enzymes tested.

Development of the PCAP primer. The PCAP primer amplified an approximately 172-bp fragment of DNA in all isolates of *P. capsici* tested from a range of hosts (Fig. 6, lanes 2 to 9 and 12; Table 1). The primer also amplified a similar-size fragment in isolates of *P. citricola* (Fig. 6, lane 11). Isolates of *P. citrophthora* were also amplified by the PCAP primer, but the amplified product was larger than that of *P. capsici* or *P. citricola* (lane 10). Digestions of the 172-bp fragment with *Msp*I differentiated *P. capsici* from *P. citrophthora* and *P. citricola*, which were not digested by this enzyme (Fig. 7). Apparently two different PCR products, both approximately 172 bp in size, were amplified by the PCAP and ITS 1 primer pair. Restriction digestion with *Msp*I yielded a 172-bp product and several smaller products in isolates of *P. capsici* (Fig. 7, lanes 2 to 9 and 12). *P. capsici* and *P. citricola* can also be differentiated by restriction digestion of ITS DNA with *Msp*I (Fig. 4, lanes 5 and 6; Table 3). None of the other species of *Phytophthora* tested, including *P. cactorum*, *P. palmivora*, *P. nicotianae*, *P. infestans*, *P. mirabilis*, *P. fragariae*, *P. sojae*, *P. megasperma*, *P. cinnamomi*, *P. cryptogea*, and *P. erythroseptica*, were amplified with the PCAP primer.

PCR was more rapid and efficient than traditional isolation methods in identification of *P. capsici* in field-infected plant samples. Of infected pepper plants with visible lesions that were positive by traditional isolation on media, 92% were also positive by PCR. The PCR method also detected 32% of the infections in samples where the pathogen was not identified previously by traditional isolation on agar media. Neither method was successful in detection of the pathogen in severely decayed tissue.

DISCUSSION

Species identification in the genus *Phytophthora* is difficult and requires the use of taxonomic keys and knowledge of the host range of the pathogen. The PCR procedures we describe in this work will provide a powerful tool for plant disease diagnosticians and researchers who are interested in the identification of many of the major species in the genus. Currently, the taxonomic key of Stamps et al. (35), which is based on

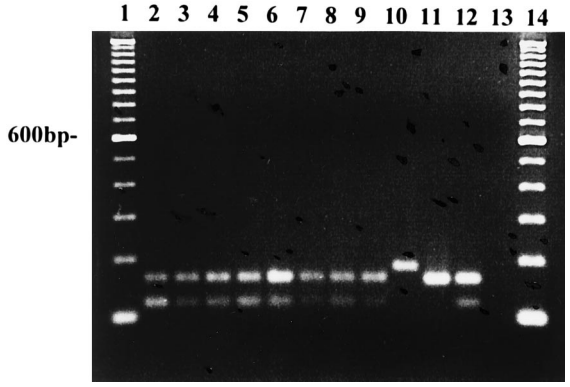

FIG. 7. Restriction digest with *Msp*I of DNA amplified with the PCAP primer and ITS 1 from *P. capsici* 17 (lane 2), *P. capsici* 18 (lane 3), *P. capsici* 19 (lane 4), *P. capsici* 20 (lane 5), *P. capsici* 21 (lane 6), *P. capsici* 22 (lane 7), *P. capsici* 23 (lane 8), *P. capsici* 25 (lane 9), *P. citrophthora* M86 (lane 10), *P. citricola* M213 (lane 11), and *P. capsici* 87 (lane 12). Lanes 1 and 14 contain 100-bp ladders; lane 13 contains a no-template control.

earlier work by Waterhouse (40), is the standard reference for identification of pathogens in the genus *Phytophthora* by classical methods. The tabular key divides the genus into six morphological groups based on characteristics of the sporangia, gametangia, growth at specific temperatures, and culture characters. We analyzed restriction fragment patterns of amplified ITS DNA from a sample of many isolates from each of six morphological groups described previously (35).

Based on morphological characteristics, isolates of *P. capsici* and *P. citricola* are placed in taxonomic groups II and III; however, these two species had common restriction fragment patterns when digested with *Rsa*I. The PCAP primer also amplified *P. capsici*, *P. citricola*, and *P. citrophthora*, indicating that there is sequence homology in the spacer I region between the 18S and 5.8S rDNA among these three species from taxonomic groups II and III. Forster et al. (11) and Cooke and Duncan (3) sequenced the ITS region I of DNA in a large number of *Phytophthora* species and identified a cluster among isolates of *P. capsici*, *P. citricola*, and *P. citrophthora* isolates. In addition, isozyme, mitochondrial DNA restriction fragment length polymorphism, and ITS DNA sequence studies have demonstrated close relationships among these three species (8, 21, 29). Lee and Taylor (21) analyzed ITS variability in several *Phytophthora* species, and their data support a close relationship between cacao isolates of *P. capsici* and *P. citrophthora* (21). The PCR primer developed by Ersek et al. (5) for *P. citrophthora* also amplified DNA of *P. capsici*. These three species have papillate or semipapillate sporangia. Our data and the data of others support the phylogenetic grouping of *P. capsici*, *P. citricola*, and *P. citrophthora* into a distinct cluster (3, 11).

Isolates of *P. nicotianae* tested from citrus, tomato, tobacco, and many ornamental plants were genetically similar after restriction digests with *Rsa*I in this amplified region of ITS DNA. Restriction fragment length polymorphism analysis of genomic DNA of this species indicated little variation among tobacco and citrus isolates (30). Hall (15) redescribed this species as a single species and suggested elimination of the forma specialis designations after extensive testing of 31 morphological, physiological, and biochemical characters (15). We did not test the host pathogenicity or physiological and biochemical traits of isolates in our work, but our data indicate little genetic variation among the isolates we tested.

P. fragariae, *P. cactorum*, *P. nicotianae*, and *P. citricola* are pathogens of strawberry plants in the United States (6, 24). These pathogens can be transported in infected propagation material and introduced into fields. *P. fragariae*, *P. cactorum*, *P. nicotianae*, and *P. citricola* are from four different taxonomic groups (V, I, II, and III, respectively) but can be easily distinguished after digestion of amplified ITS DNA with *Rsa*I. In addition, the PINF primer we developed in related work for the potato and tomato late blight pathogen *P. infestans* also amplifies *P. cactorum* (38), and the PCAP primer described in our present work for *P. capsici* amplifies *P. citricola*. Primer sequences which amplify *P. fragariae* isolates from strawberry, *P. fragariae* var. *rubi* from raspberry, and *P. nicotianae* have been reported (4, 5, 33, 34). Both specific and universal PCR primers could be used to screen plant material and improve the detection of all the major *Phytophthora* pathogens of strawberry. Strawberry plants are vegetatively propagated, and *Phytophthora* species can spread readily in infected plant material.

A number of *Phytophthora* species, including *P. nicotianae*, *P. citrophthora*, *P. palmivora*, *P. citricola*, *P. syringae*, and *P. hibernalis*, infect citrus (14, 42). Four of these six species were examined in this study. *P. nicotianae* and *P. citrophthora* are the two most common species on citrus, and they were easily distinguished after restriction digestion with *Rsa*I. *P. citrophthora* and *P. nicotianae* both cause gummosis and root rot of citrus, but *P. citrophthora* is more active in the fruit and aerial plant parts than *P. nicotianae*. The incidence of brown rot on fruit caused by *P. palmivora* has increased in recent years in Florida (14a).

There was variation in restriction fragment patterns of ITS DNA among the group V isolates of *P. megasperma*. Variation within this species has also been noted by others (7, 10, 11, 16, 17, 30, 43). The host-specialized form species of isolates that infect legumes within *P. megasperma* have been given species designations and include *P. sojae*, *P. medicaginis*, and *P. trifolii* (17). However, the isolates of *P. megasperma* from woody hosts were placed into the broad-host-range (BHR) lineage and separated into electrophoretic types BHR, AC, and DF karyotypes (16, 17). Sequence differences in the ITS spacer I region indicate that the pathogens in this BHR group do not represent a single biological species (11). In our work, two isolates of *P. megasperma* from peach and walnut had restriction patterns that differed from the other isolates from raspberry, apricot, and cherry. One of these isolates from peach (NY 412) was studied previously by others and is the A/C electrophoretic type sensu Hansen et al. (17, 42a). This isolate is in a morphologically, culturally, and electrophoretically distinct group that has been referred to as the small-oospore, high-temperature type group of *P. megasperma* (44). Further work must be done with a larger number of isolates of *P. megasperma* from the woody-host group to further delineate fruit tree isolates. Separate species designations for the fruit tree isolates are probably warranted, as has been done with the legume isolates (7), to clarify the taxonomy of *Phytophthora megasperma*.

P. infestans, *P. mirabilis*, and *P. cactorum* had identical restriction fragment patterns when their ITS DNA was digested with *Rsa*I. These three species also yielded an identical product when amplified with the PINF primer (38). Others also developed a PCR primer that amplifies both *P. infestans* and *P. mirabilis* and found similarities in the ITS region II between these species (36, 37). *P. cactorum* can be easily differentiated from *P. infestans* after restriction digests with *Msp*I. The ITS region I DNA sequences of *P. cactorum* and *P. infestans* were similar, and these species also formed a cluster in phylogenetic analysis (3, 11). In our work, *P. mirabilis* and *P. infestans* were not distinguishable after restriction digestion with a number of enzymes including *Rsa*I, *Msp*I, *Eco*RI, and *Hae*III. These data suggest that the two species have considerable sequence homology in this region of ITS DNA. *P. mirabilis* was first described as a new species on *Mirabilis jalapa* in Mexico in 1985 (12). Other authors have suggested that *P. mirabilis* should be called a forma specialis of *P. infestans* (26). *P. infestans* and *P. mirabilis* have similar mitochondrial DNA restriction patterns (26). An oligonucleotide probe, pL121-3, was developed to differentiate *P. mirabilis* from *P. infestans*, but the reaction of the probe with other potato pathogens was not examined (26). We have not yet sequenced the ITS DNA of *P. mirabilis*, but our data also suggest that two species designations may be unwarranted.

It is evident from our work and that of others (3, 11, 28, 29) that the morphological differentiations of the major species of *Phytophthora* do not necessarily represent genetic differences among the species. Isolates with divergent sporangial characters, temperature requirements, and hosts have sequence homology in their ITS DNA. The isolates used in our present study were well characterized by morphological criteria before the advent of PCR. Identification by classical methods requires expertise and is time-consuming. The molecular methods we describe will provide useful and rapid tools for identification of

the economically important species within this plant-pathogenic genus.

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