Cloned DNA Probes for Identification of *Phytophthora parasitica*

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ABSTRACT

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Chromosomal DNA of *Phytophthora parasitica*, digested with *Hin*dIII and *Eco*RI, was ligated to pUC8 and used to transform *Escherichia coli*. Three recombinant plasmids were identified that hybridized to *P. parasitica* DNA but not to DNA of other *Phytophthora* species, healthy tomato roots, and *Pythium* species. DNA from all isolates of *P. parasitica* tested, including *P. parasitica* var. *nicotianae*, hybridized strongly with the probes. Southern blot hybridizations revealed that the probes hybridized to multiple restriction fragments, suggesting that the cloned sequences recognize repetitive DNA. There was no homology between the three cloned DNA fragments of *P. parasitica*. A non-radioactively labeled DNA probe, chemically modified by sulfonation, was as sensitive as a ^{32}P-labeled DNA probe for the detection of purified *P. parasitica* DNA. These species-specific DNA probes provide a sensitive new tool for the identification of *P. parasitica*.

Phytophthora parasitica Dast. causes diseases of many plants, including important root and fruit rots of citrus and tomato (15). Many *Phytophthora* species have wide host ranges, and it is common for several species to cause similar symptoms on a single host. For example, *P. parasitica*, *P. citrophthora*, and other *Phytophthora* spp. can cause brown rot of citrus, and although *P. parasitica* causes the vast majority of root rot of processing tomatoes in California, *P. capsici* also causes this disease (11,17). Pathogenicity on a particular host, therefore, is not a satisfactory characteristic for species identification; however, it is frequently necessary for researchers and diagnosticians to correctly identify which species of *Phytophthora* is causing a particular disease.

Identification and classification of *Phytophthora* spp. is based on the morphology of fungal structures, which are typically examined in pure culture (20). For many *Phytophthora* species, however, variability in morphology within and between species is often too large to allow reliable identification (5). Many taxonomic characteristics have continuous rather than discrete variation, and a large proportion of isolates can differ considerably from the type species (1,5). Because many important taxonomic characteristics have ranges that overlap between species, isolates are frequently placed into a gray area between species (5). The result is often the description of atypical isolates, which must later be reassigned to a different species, or the separation of variants of one species into several species. Accurate identification of an isolate of *Phytophthora* to species requires considerable time and experience.

While morphology will likely continue to be important in the taxonomy of *Phytophthora*, less ambiguous and difficult criteria would be helpful in identification. Cloned random DNA fragments have been used as probes to identify species of fungi, such as *Fusarium oxysporum* and *Phoma tracheiphila* (14,16). There are several advantages to identifying fungi with a species-specific DNA probe. Pure cultures are unnecessary because cloned probes can be selected for their inability to hybridize to DNA from other fungi. The need to produce characteristic fungal structures in culture, such as antheridia and oogonia, is eliminated because DNA can be extracted from any living mycelium. DNA probes are also highly sensitive and can routinely detect as little as 1 ng of DNA (4). Species identification with a DNA probe can be relatively rapid and provides a degree of objectivity not always possible in traditional methods of *Phytophthora* identification.

The purpose of the research reported here was to identify and characterize random cloned DNA fragments that are specific to *P. parasitica*. These DNA probes should have future applications in detection of the fungus in the greenhouse and field.

MATERIALS AND METHODS

Fungal isolates. Tested isolates of *Phytophthora* and *Pythium* species and their sources are listed in Table 1. Cultures were stored on corn meal agar slants at 25 C.

TABLE 1. Hybridization of DNA probes to isolates of *Phytophthora* and *Pythium* spp.

Isolate and source[z]		pPP28	pPP33	pPP34
Phytophthora parasitica				
5-3A	tomato, Yolo Co., CA[c]	+	+	+
W-1	tomato, Yolo Co., CA[c]	+	+	+
C-2CL	citrus, AZ[c]	+	+	+
MiS-22C	jojoba, AZ[c]	+	+	+
34-3-9	pistachio, CA[c]	+	+	+
37-4-1	jojoba, CA[c]	+	+	+
30-2	tomato, Yolo Co., CA[c]	+	+	+
30-2DM	tomato, Yolo Co., CA[c]	+	+	+
1-3A	tomato, Yolo Co., CA[c]	+	+	+
P. parasitica var. *nicotianae*				
1452	tobacco, KY[f]	+	+	+
CII	tobacco, Pakistan[c]	+	+	+
P. cactorum				
ATCC58007	walnut, Yolo Co., CA[d]	−	−	−
18-4-4	safflower, CA[c]	−	−	−
Ap216C	apple, San Joaquin Co., CA[c]	−	−	−
P. cambivora				
3-2-5	apple, OR[d]	−	−	−
9-2-1	Japanese maple, OR[d]	−	−	−
12-4-5	apple, CA[d]	−	−	−
P. capsici				
ATCC15399	pepper, CA[c]	−	−	−
CI	pepper, Pakistan[c]	−	−	−
1787	soil, CA[a]	−	−	−
SC2B	pepper, NC[c]	−	−	−
26-2-7	pumpkin, Chile[c]	−	−	−
1794	soil, CA[a]	−	−	−
P. cinnamomi				
SB216-1	avocado, CA[d]	−	−	−
4-3-3	camillia, CA[c]	−	−	−
P. citricola				
ATCC58009	walnut, Butte Co., CA[d]	−	−	−
1-1-3	lilac, Canada[d]	−	−	−
21-1-1	walnut, Butte Co., CA[d]	−	−	−
SG3-20	river water, Stanislaus, CA[d]	−	−	−
P. citrophthora				
P1323	citrus, CA[b]	−	−	−
18-4-8	walnut, Chile[d]	−	−	−
P1201	cacao, Brazil[f]	−	−	−
34-4-5	cherry, CA[d]	−	−	−
32-4-7	walnut, Chile[d]	−	−	−
ATCC13613	citrus, CA[d]	−	−	−
P318	citrus, Australia[f]	−	−	−
14A	kiwi, Sutter Co., CA[b]	−	−	−
P. cryptogea				
RI	safflower, AZ[c]	−	−	−
P201	safflower, AZ[c]	−	−	−
13-4-9	cherry, CA[d]	−	−	−
P. erythroseptica				
PO-1	potato, CA[d]	−	−	−
P. hibernalis				
32-4-3	lemon, CA[c]	−	−	−
P. megasperma				
5-58	soybean, IL[c]	−	−	−
pH3	alfalfa, Yolo Co., CA[c]	−	−	−
P-74-R	peach, Yuba Co., CA[d]	−	−	−
Pythium aphanidermatum				
72-13	alfalfa, CA[g]	−	−	−
86-10	alfalfa, CA[g]	−	−	−
P. irregulare				
83-6	alfalfa, CA[g]	−	−	−
86-10	alfalfa, CA[g]	−	−	−
P. ultimum				
ATCC32929	cotton, CA[a]	−	−	−
67-1	cotton, CA[g]	−	−	−
82-28	alfalfa, CA[g]	−	−	−

[z]Obtained from culture collections of a) E. E. Butler, b) K. Conn, c) J. M. Duniway, and d) S. M. Mircetich, Department of Plant Pathology, University of California, Davis; e) M. E. Matheron, Yuma Mesa Agricultural Center, Somerton, AZ; f) M. D. Coffey, Department of Plant Pathology, University of California, Riverside; g) J. G. Hancock, Department of Plant Pathology, University of California, Berkeley.

DNA isolation. Agar plugs containing fungal mycelium were used to inoculate 25 ml of lima bean broth (3). After 3 days at 25 C, the broth cultures were homogenized for 30 sec in a sterile blender and then used as inoculum for 100 ml of lima bean broth amended with 250 μg of ampicillin per milliliter and 10 μg of rifampicin per milliliter to prevent bacterial contamination. Following 4 days of shake culture (25 C, 100 rpm), mycelium was harvested by filtering through Miracloth, rinsed with sterile distilled H_2O, and lyophilized. Lyophilized mycelium was stored at −20 C.

To extract DNA, 2–8 g of lyophilized mycelium was ground to a fine powder with a mortar and pestle and then suspended in 100 ml of extraction buffer (20 mM Tris, 100 mM NaCl, 20 mM EDTA, 0.2% 2-mercaptoethanol [v:v], pH 8.0). To this suspension, 10 ml of 10% *N*-lauryl sarcosine and 50 ml of phenol:chloroform (1:1) were added, and the suspension stirred for 1 hr at 4 C. The suspension was then centrifuged for 10 min at 10,000 *g*, and the aqueous phase was extracted with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA in the aqueous phase was precipitated with a tenth volume of 3 M sodium acetate (pH 5.4) and 2 volumes of cold ethanol and centrifuged at 14,000 *g* for 30 min (13). The precipitate was redissolved in 6 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and then sequentially extracted with an equal volume of phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1). DNA in the aqueous phase was then precipitated by adding two-thirds volume of 5 M ammonium acetate and 2 volumes of cold ethanol and centrifuged at 14,000 *g* for 30 min (13). The precipitate was suspended in 6 ml of TE and dialyzed overnight against three changes of TE. The DNA was then centrifuged in CsCl gradients with bisbenzimide according to Hudspeth et al (8) to separate chromosomal from mitochondrial DNA. Chromosomal DNA was dialyzed in TE and stored at −20 or 4 C. Healthy tomato roots were frozen with liquid N_2, ground to a fine powder with a mortar and pestle, and the chromosomal DNA was then purified by the procedure described above.

Small amounts of DNA (200–1,000 ng) of many of the isolates listed in Table 1 were isolated using a modified miniprep procedure (21). Aerial mycelium was removed from 7–10-day-old cultures of *Phytophthora* or *Pythium* grown on V-8 juice agar. The mycelium was frozen with liquid nitrogen and ground to a fine powder in a 1.5-ml microfuge tube. To this was added 0.5 ml of miniprep extraction buffer (100 mM Tris, 40 mM EDTA, 0.2% SDS, 0.2% 2-mercaptoethanol, pH 8.0). The remainder of the procedure was unchanged from the published procedure (21), except that the DNA preparation was extracted with phenol:chloroform (1:1) and then chloroform:isoamyl alcohol (24:1) before DNA precipitation with isopropanol, and only one DNA precipitation was performed.

Construction of DNA library and probe preparation. Standard DNA manipulations were performed according to Maniatis et al (13), unless otherwise stated. Chromosomal DNA of *P. parasitica* 5-3A was digested with *Hin*dIII and *Eco*RI, and the resulting fragments were size fractionated by electrophoresis in 1% agarose TBE gels (0.089 M Tris, 0.089 M boric acid, 0.02 M EDTA). Fragments of approximately 0.55 to 3.2 kb were recovered from the gel with DE 81 cellulose paper (2), ligated into *Hin*dIII and *Eco*RI digested pUC8 (19), and used to transform competent cells of *Escherichia coli* DH5α. Replicate plates of ampicillin-resistant colonies grown on nitrocellulose membranes were screened by colony hybridization to *P. parasitica* 5-3A or *P. citrophthora* P1323 chromosomal DNA labeled with ^{32}P-dATP to a specific activity of 10^8 cpm/μg by the oligolabeling procedure (6). Clones that hybridized to ^{32}P-labeled-DNA of *P. parasitica* 5-3A but not to that of *P. citrophthora* P1323 were selected, grown in LB broth (13), and plasmid DNA was isolated by an alkaline minipreparation procedure (18). Plasmid DNA was diluted in 6× SSC (1× SSC = 0.015 M sodium citrate, 0.15 M sodium chloride) boiled 5 min, quickly cooled, and applied to a nitrocellulose membrane (BA45, Schleicher and Schuell) with a dot blot manifold (Hybri-dot manifold, Bethesda Research Laboratories, Gaithersburg, MD). The dot blots were hybridized

with ^{32}P-labeled DNA of *P. parasitica* or *P. citrophthora*. Three plasmids, designated pPP28, pPP33, and pPP34, which hybridized to *P. parasitica* DNA, were labeled with ^{32}P-dATP as previously described, and hybridized to dot blots containing 10–80 ng of DNA from healthy tomato roots, and fungi listed in Table 1. Restriction enzyme mapping of plasmid DNA was performed by sequential digestions (13).

Southern blots. For Southern blots, chromosomal *Phytophthora* DNA was further purified by DEAE chromatography (13). Approximately 0.75 μg of DNA per lane, digested with *Hin*dIII and *Eco*RI, was electrophoresed in 1% agarose TBE gels. The gels were stained with ethidium bromide, transferred to nitrocellulose membranes as described by Maniatis et al (13), and hybridized as previously described.

All membranes were baked at 80 C for 2 hr under vacuum, prehybridized and hybridized under the conditions described by Kirkpatrick et al (12). After hybridization, membranes were exposed to XAR X-ray film (Kodak) for 3 hr to 7 days at −70 C with intensifying screens (Lightning Plus, Du Pont).

Chemically labeled DNA probes. Fungal and plant DNA was isolated and applied to a nylon membrane (Nytran, Schleicher and Schuell) with a dot blot manifold as previously described. Plasmid pPP33 was sulfonated as described by the manufacturer (Chemiprobe, FMC Bioproducts, Rockland, ME). Prehybridization and hybridization were performed as previously described using 50 ng of sulfonated plasmid DNA per square centimeter of membrane. Immunological visualization of the bound probe was performed using a monoclonal antibody that was specific to sulfonated DNA, and an alkaline phosphatase anti-mouse immunoglobulin conjugate according to the manufacturer's instructions. Color development was halted after 7–10 min of incubation with substrate (nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate).

RESULTS

Identification of *P. parasitica*-specific DNA probes. Nine hundred ampicillin-resistant colonies were screened by colony hybridization. Three recombinant plasmids, pPP28, pPP33, and pPP34, hybridized only to DNA of *P. parasitica*. No detectable hybridization occurred between these plasmids and DNA from healthy tomato roots, DNA from three different *Pythium* spp. which cause root rots of many plants, and DNA from 35 different isolates of other *Phytophthora* spp., including *P. capsici*, which also infects tomatoes (Fig. 1 and Table 1). All isolates of *P. parasitica* tested, obtained from a wide variety of hosts and locations, hybridized similarly with pPP33 (Fig. 1 and Table 1). With ^{32}P-labeled probes, the minimum amount of DNA detectable in dot blot hybridizations was approximately 10 to 1 ng of purified *P. parasitica* DNA (Fig. 1). Identical results were obtained when pPP28 and pPP34 were used as probes and are summarized in Table 1. No significant hybridization occurred between DNA of any of the *Phytophthora* spp. and the plasmid vector, pUC8 (data not shown).

Restriction sites and Southern blot analysis of *P. parasitica*-specific DNA probes. Digestion of pPP28 and pPP34 with *Hin*dIII and *Eco*RI excised single fragments of *P. parasitica* DNA with sizes of 0.56 and 0.57 kb, respectively (Fig. 2A and C). However, two fragments (1.11 and 1.94 kb) of *P. parasitica* DNA were observed when pPP33 was similarly digested (Fig. 2B). A restriction map of pPP33 (Fig. 3), shows that three *Hin*dIII restriction sites are present, but two of the sites, located at the ends of the cloned insert, are associated with the multiple cloning site polylinker of pUC8. It appears that two polylinkers flank the cloned DNA fragment of *P. parasitica*. Evidence for the existence of two polylinkers was the presence of pUC8 as a single fragment of 2.7 kb following digestion of pPP33 with *Eco*RI, *Sma*I, *Bam*HI, *Sal*I, *Hin*cII, *Pst*I, or *Hin*dIII. The polylinker of pUC8 is composed of the recognition sequences for these enzymes, and is delimited by the *Eco*RI and *Hin*dIII sites. It is more likely that these sequences are present because there are two polylinkers in pPP33 rather than recognition sequences resembling the polylinker of pUC8 in the ends of the cloned DNA fragment of *P. parasitica*. The result is that digestion of pPP33 with *Eco*RI alone releases the entire insert of *P. parasitica* DNA (lane 7, Fig. 2B), and digestion with *Hin*dIII alone produces similar fragments as the *Hin*dIII/*Eco*RI digestion but the smaller insert fragment is slightly larger (1.14 versus 1.11 kb), because it includes polylinker DNA (lane 6, Fig. 2B). The total length of the insert, including polylinker DNA, is 3.1 kb for pPP33.

Southern blots of *Hin*dIII- and *Eco*RI-digested *P. parasitica* DNA showed numerous DNA bands that hybridized with pPP28, pPP33, or pPP34 (Fig. 2). No detectable homology was observed in Southern blots when similarly digested DNA of *P. cactorum* and *P. citrophthora* were probed with these plasmids (data not shown). The insert DNA of all three probes hybridized to restriction fragments of *P. parasitica* chromosomal DNA that were the same size as the insert DNA, as well as with numerous larger and smaller fragments (lanes 1–4, Fig. 2A–C). An indication that the chromosomal DNA of the Southern blots was completely digested was provided by the presence of a chromosomal DNA fragment corresponding in size to the 1.11-kb *Hin*dIII/*Eco*RI fragment of pPP33 rather than to the slightly larger fragment digested with *Hin*dIII alone (lanes 6 and 8, Fig. 2B). No homology

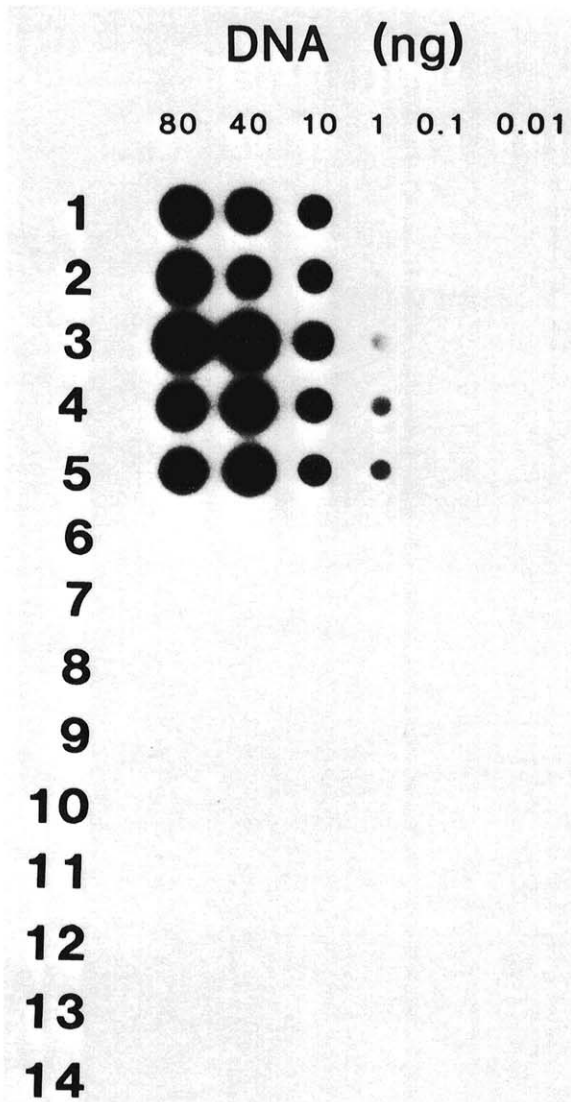

Fig. 1. Dot hybridization of pPP33 to fungal and plant DNA. 1 = *Phytophthora parasitica* 5-3A, 2 = *P. parasitica* W-1, 3 = *P. parasitica* C-2CL, 4 = *P. parasitica* 1452, 5 = *P. parasitica* MiS-22C, 6 = *P. cactorum* ATCC58007, 7 = *P. cinnamomi* SB216-1, 8 = *P. citricola* ATCC58009, 9 = *P. citrophthora* P1323, 10 = *P. cryptogea* RI, 11 = *P. megasperma* P-74-R, 12 = *P. erythroseptica* PO-1, 13 = *Pythium ultimum* ATCC32929, 14 = *Lycopersicon esculentum* roots.

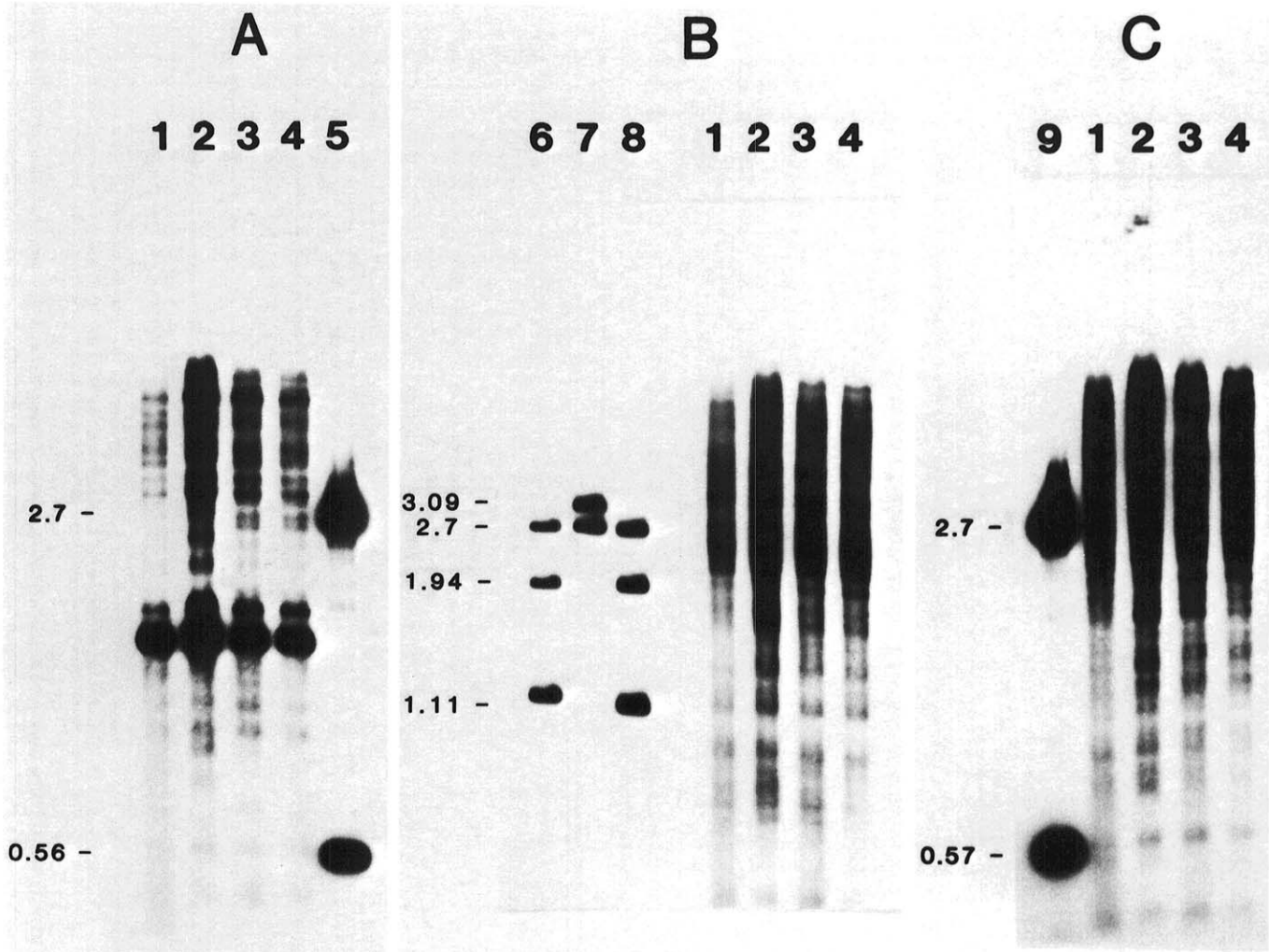

Fig. 2. Southern hybridization of *Phytophthora parasitica* DNA and plasmid probe DNA. All DNAs were doubly digested with *Hin*dIII, and *Eco*RI, except lane 6, which was digested with *Hin*dIII, and lane 7, which was digested with *Eco*RI. A = pPP28 probe. B = pPP33 probe. C = pPP34 probe. Plasmid fragment of 2.7 kb hybridizing with the DNA probes was pUC8. 1 = *P. parasitica* MiS-22C, 2 = *P. parasitica* C-2CL, 3 = *P. parasitica* W-1, 4 = *P. parasitica* 5-3A, 5 = pPP28, 6 = pPP33, 7 = pPP33, 8 = pPP33, 9 = pPP34.

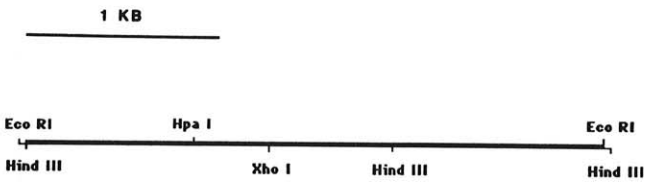

Fig. 3. Restriction enzyme map of the cloned insert in pPP33. The wide horizontal line indicates DNA of *Phytophthora parasitica*. The narrow horizontal line indicates DNA of the multiple cloning site polylinker of pUC8. Length of *P. parasitica* DNA fragment is 3.0 kb. Total length of *P. parasitica* and polylinker DNA is 3.1 kb.

was observed between the different cloned inserts of *P. parasitica* DNA when pPP28, pPP33 and pPP34, digested with *Hin*dIII and *Eco*RI, were hybridized to each other in Southern blot analysis (data not shown).

Detection of fungal DNA with sulfonated probes. The sensitivity and specificity of the sulfonated DNA probe in detecting purified *P. parasitica* DNA was similar to that of the ^{32}P-labeled probe (compare Figs. 1 and 4). Approximately 1 ng of *P. parasitica* DNA was detected without significant hybridization to purified DNA from other *Phytophthora* spp. and *Pythium ultimum* (Fig. 4). Significant amounts of cross reactivity occurred with certain *Phytophthora* spp. other than *P. parasitica* if the chromogenic substrate was allowed to remain on the membrane for longer than 7–10 min. The nature of this cross reactivity is unclear, but it is not due to alkaline phosphatase activity in the purified DNA preparations, which was determined to be negligible.

DISCUSSION

The cloning and identification of DNA probes specific to *P. parasitica* provides an important new tool for unambiguous detection and identification of a member of this taxonomically complex genus. The three probes described here should permit the identification of most, if not all, isolates of *P. parasitica*. The probes hybridized equally well to DNA of *P. parasitica* isolated from a variety of hosts and geographical locations including that of *P. parasitica* var. *nicotianae*, which differs from *P. parasitica* in its ability to cause a disease of tobacco at any stage of growth (7). The three DNA probes were also highly species-specific. No hybridization was observed between the probes and purified DNA of several other *Phytophthora* spp., including *P. citrophthora*, which has very similar morphological characteristics to *P. parasitica* (7). Considering the number of taxonomically related fungi whose DNA did not hybridize with the probes, it is relatively unlikely that the probes will be found to hybridize with DNA of other more distantly related fungal species.

The multiple banding patterns observed in Southern blots of *P. parasitica* DNA probed with the species-specific plasmids were not completely unexpected. Probes were selected for the maximum

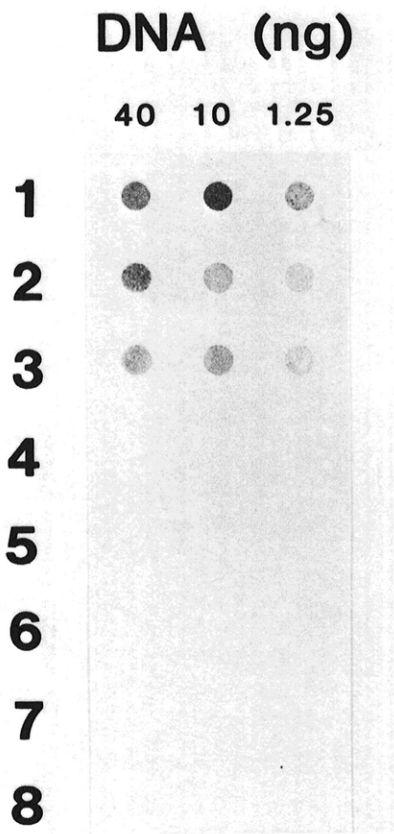

Fig. 4. Dot hybridization of sulfonated pPP33 probe to DNA of *Phytophthora parasitica* and other fungi. 1 = *P. parasitica* 5-3A, 2 = *P. parasitica* W-1, 3 = *P. parasitica* C-2CL, 4 = *P. cactorum* ATCC58007, 5 = *P. cinnamomi* SB216-1, 6 = *P. citrophthora* P1323, 7 = *P. erythroseptica* PO-1, 8 = *Pythium ultimum* ATCC32929.

intensity of hybridization to *P. parasitica* DNA, which would also tend to select for clones containing medium to high copy number sequences of *P. parasitica* DNA. The advantage of such probes is that the detection signal is multiplied because there are multiple copies of the target sequence, and therefore these probes should provide greater sensitivity in soil and plant assays than a probe hybridizing to a single copy sequence. In addition, a multiple copy sequence probe should be more reliable because mutations in one copy of a high copy sequence would only slightly affect the total amount of hybridization.

Other studies have demonstrated the advantage of a DNA probe recognizing multiple copy sequences. Random DNA probes developed to detect *Phoma tracheiphila*, which were not selected to recognize repetitive sequences, were not sensitive enough to detect the pathogen in practical applications (16). Other clones, however, were selected for maximum hybridization signals to labeled *P. tracheiphila* DNA, and a probe was obtained that hybridized to a highly repetitive sequence, which was specific to *P. tracheiphila*. This probe was sufficiently sensitive to detect the pathogen in infected plant tissues under laboratory and field conditions (16).

The hybridization patterns of the southern blots indicate that the cloned *P. parasitica* DNA of the probes is repetitive DNA, or possibly, but less likely, DNA of transposable elements or multigene families. In *Achyla bisexualis*, an oomycete, repetitive DNA comprises 16% of the chromosomal DNA with perhaps over half of the repetitive DNA consisting of multigene coding sequences (9). The genome of *Bremia lactucae*, which is more closely related to *Phytophthora*, contains as much as 65% repetitive DNA, which is distributed in a short interspersion pattern, and approximately one-third of the repetitive DNA is present in 1,000 or more copies (D. Francis, S. Hulbert, and R. Michelmore, *personal communication*). If the genome of *P. parasitica* is similarly constructed, then the likelihood of cloning a random fragment containing repetitive DNA would be relatively high. In a study of restriction fragment length polymorphisms of *Fusarium* spp., a DNA probe that hybridized with multiple restriction fragments in Southern blots was also specific to a set of formae speciales of *F. oxysporum* (14). It was proposed that the probe did not recognize repetitive ribosomal-encoding DNA that was presumed to be conserved throughout the genus, but instead recognized a gene family peculiar to this set of formae speciales (14).

Modification of the *P. parasitica* DNA probe by sulfonation with the Chemiprobe system detected 1 ng of DNA with negligible nonspecific background reactions. Similar results were obtained when sulfonated DNA probes were used to detect DNA from *Mycoplasma* spp. (10). For the detection of *Mycoplasma* DNA, biotinylated DNA probes had a similar sensitivity to sulfonated probes but produced greater nonspecific reactions than the sulfonated probes (10). The development of non-radioactive DNA labeling should expand the usefulness of *Phytophthora* species-specific probes.

The potential uses of species-specific DNA probes in plant pathology are many. Unambiguous and reliable detection of fungal pathogens such as *Phytophthora* spp. is critical in disease diagnosis and in ecological and epidemiological research. The *P. parasitica*-specific DNA probes can detect the fungus in infected plant tissue and have been adapted to detect colonies of *P. parasitica* developing from infested soil samples (P. H. Goodwin, *unpublished*). Once species-specific DNA probes have been developed to the most common *Phytophthora* species, it should be possible to identify most isolates, and it should be simpler to study disease complexes involving several *Phytophthora* spp.

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