Detection of *Phytophthora* Species by Oligonucleotide Hybridization to Amplified Ribosomal DNA Spacers

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ABSTRACT

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Four probes were developed to distinguish DNA from isolates of *Phytophthora capsici, P. cinnamomi, P. megakarya,* and *P. palmivora.* These four probes complement different ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequences that exhibit variations between species but not within species, based on previous comparative DNA-sequence analyses of 18 *P. cinnamomi*, two *P. palmivora*, two *P. megakarya*, and two *P. capsici* isolates (21). A fifth probe that was complementary to identical sequences in all *Phytophthora* isolates tested, a "genus *Phytophthora*" probe, was developed. DNA-DNA hybridization of the probes to ITS amplified by polymerase chain reaction (PCR) from 30 isolates representing seven *Phytophthora* species and from 12 isolates representing nine other genera of the Oomycete class demonstrated the utility of this approach. Probes of *P. capsici, P. cinnamomi, P. megakarya,* and *P. palmivora* hybridized only to their respective targets: three isolates of *P. capsici*, 18 isolates of *P. cinnamomi*, one isolate of *P. megakarya*, and two isolates of *P. palmivora.* In addition, the "genus *Phytophthora*" probe hybridized to the target DNA of all 30 isolates of *Phytophthora* species tested but not to DNA of isolates from nine other Oomycete genera.

Traditional taxonomy in *Phytophthora* is based primarily on growth characteristics and morphology (18,27,30,31) and has always been difficult (4,9). This difficulty results from the limited number of morphological characteristics available for species identification and the variability of these characteristics (4,9). In order to accurately identify many species, it often is necessary to perform statistical analyses on highly variable, quantitative features of both asexual and sexual fungal stages produced in pure culture (25,30). Other characteristics used for diagnosis include cardinal temperatures for growth, the number and size of chromosomes, and host-pathogenicity assays (9). In combination, these methods can produce accurate identification; however, they are time-consuming, tedious, and can be difficult to interpret (4).

Rapid, simple, and reliable identification of *Phytophthora* species may be possible using molecular techniques (11–13,26). Species-specific cloned DNA probes have been used to identify isolates of *P. parasitica* and *P. citrophthora* (11–13). Repetitive DNA polymorphism analysis (26) has been used for the identification of isolates of *Phytophthora* to species. Serological assay kits also have been tested for their ability to detect *P. cinnamomi* (3). These techniques are limited by either tedious and costly development of probes, by exhibiting variation at the subspecific level, or by being founded on characteristics of unknown origin.

In the present report, we have evaluated the use of oligonucleotide DNA probes designed to distinguish DNA from isolates of *P. capsici* (Leonian), *P. cinnamomi* (Rands), *P. megakarya* (Brasier & Griffin), and *P. palmivora* (E.J. Butler) E.J. Butler. These four probes complement different ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequences that exhibit variation among *Phytophthora* species. Variation within species was undetected in the ITS sequences used to design probes for two *P. capsici*, 18 *P. cinnamomi*, two *P. megakarya*, and two *P. palmivora* isolates (21). ITS was also chosen because it is a well-studied molecule, it is present in high-copy numbers, and it is easily isolated by polymerase chain reaction (PCR) (32). Another probe that is complementary to sequences present in all the *Phytophthora* isolates previously sequenced (21), called the "genus *Phytophthora*" probe, was developed. The application of a characterized DNA probe coupled with PCR amplification for identification has been demonstrated previously with fungi (10,16,28), yeast (15), bacterial species (2,7), and other microorganisms (1) but not with phytopathogenic Oomycete species.

MATERIALS AND METHODS

Isolates and sources. The source and the collection number for isolates of *Phytophthora, Pythium, Pythiogeton, Peronospora, Albugo, Bremia, Aqualinderella, Mindeniella, Leptomitus,* and *Lagenidium* are listed in Table 1.

Growth condition. *Phytophthora* isolates were maintained on slants of V8-C agar, prepared by adding 345 ml of cheesecloth-filtered V8 juice, 5 g of $CaCO_3$, 1,380 ml of distilled water, and 15 g of agar per liter (27). V8-C broth was prepared in the same way, with the exclusion of agar (27). Isolates were transferred to either V8-C agar petri plates or V8-C broth plates (15 ml per plate), which were incubated for 2–4 days at 20 C, and mycelium for DNA extraction, ~0.05 g dry weight, was taken from 2- to 4-cm-diameter colonies. Cultures of all other isolates were kindly provided by the sources listed in Table 1.

DNA extraction. Colonies scraped off agar or harvested from V8-C broth by vacuum filtration through cheesecloth (2- to 4-cm-diameter) were immediately frozen in liquid nitrogen or were lyophilized. Frozen or lyophilized mycelia were ground into a fine powder in a mortar and pestle. Conidia of the obligate plant-pathogen *Albugo candida* were isolated from cabbage leaves by gently scraping sori from the undersides of infected leaves. DNA from mycelium or spores was extracted by the methods of Lee, Milgroom, and Taylor (19) or Lee and Taylor (20). DNA of *Bremia lactucae* and *Peronospora parasitica* was kindly provided by Richard Michelmore (U. of California, Davis).

PCR amplification. Ribosomal ITS fragments were amplified

TABLE 1. Hybridization of oligonucleotide probes to target DNA from isolates used in this study

Species	Isolate number[b]	Mating type	Host association, origin/source	Oligonucleotide probe[a]					
				all	PH	pl	cap	mg	cin
P. cinnamomi	a119	A1	*Castanopsis* sp., PNG	+	+	−	−	−	+
	a125	A1	*Nothofagus* sp., soil, PNG	+	+	−	−	−	+
	p2100	A1	*Camelia* sp., CA	+	+	−	−	−	+
	p2121	A1	*Persea americana*, CA	+	+	−	−	−	+
	p2159	A1	grape, S Af.	+	+	−	−	−	+
	p2264	A1	*Yucca* sp., Australia	+	+	−	−	−	−
	p2110	A2	*Cinnamomum* sp., Sumatra	+	+	−	−	−	+
	p2144	A2	*Azalea* sp., Ohio	+	+	−	−	−	+
	p2213	A2	soil trap, New Zealand	+	+	−	−	−	+
	p2288	A2	*Pinus radiata*, CA	+	+	−	−	−	+
	p2411	A2	*Juglans* sp., CA	+	+	−	−	−	+
	a2423	A2	*P. radiata*, NSW	+	+	−	−	−	+
	p2472	A2	*Persea americana*, CA	+	+	−	−	−	+
	p2540	A1	*Leucodendron*, S Af.	+	+	−	−	−	+
	p6340	A2	*Pinus kesiya*, PNG	+	+	−	−	−	+
	p6341	A2	*Rhododendron*, PNG	+	+	−	−	−	+
	p6343	A2	rubber plantation, PNG	+	+	−	−	−	+
	p6349	A2	*P. radiata*, needle bait, NSW	+	+	−	−	−	+
P. palmivora	p255	A2	*Theobroma cacao*, Costa Rica	+	+	+	−	−	−
	p551	A1	*Theobroma cacao*, Jamaica	+	+	+	−	−	−
P. capsici	p622	A1	*Theobroma cacao*, Brazil	+	+	−	+	−	−
	p623	A2	*Theobroma cacao*, Brazil	+	+	−	+	−	−
	GA37	...	R. Specker	+	+	ND	ND	ND	ND
	p864	A1	*Theobroma cacao*, Cameroon	+	+	ND	ND	ND	ND
P. megakarya	p1664	A1	*Theobroma cacao*, Nigeria	+	+	−	−	+	−
P. citrophthora	p449	...	*Theobroma cacao*, Brazil	+	+	−	−	−	−
P. cactorum	148	...	R. Specker	+	+	ND	ND	ND	ND
P. megasperma	5-58	...	soybean, M. Hahn	+	+	ND	ND	ND	ND
P. gonapodyides	GA25	...	R. Specker	+	+	ND	ND	ND	ND
Pythium aphanadermatum	51-018	...	J. Middleton	+	−	−	−	−	−
Pythium oligandrum	51-020	...	J. Middleton	+	−	−	−	−	−
Pythium spinosum	GA33	...	R. Specker	+	−	−	−	−	−
Pythiogeton	CRB11	...	R. Emerson, Costa Rica	+	−	−	−	−	−
Peronospora parasitica[c]	...	...	R. Michelmore	+	−	−	−	−	−
Albugo candida	...	...	R. Raabie, CA	+	−	−	−	−	−
Bremia lactucae[c]	SF5	...	R. Michelmore	+	−	−	−	−	−
Bremia lactucae[c]	C82P24	...	R. Michelmore	+	−	−	−	−	−
Aqualinderella fermentans	CRB 30	...	R. Emerson, Costa Rica	+	−	−	−	−	−
Mindeniella spinospora	54-011	...	H. Whisler, MS	+	−	−	−	−	−
Leptomitus lacteus	59-004	...	H. Whisler, Augusta, GA	+	−	−	−	−	−
Lagenidium chthlamilophilon	65-028	...	M. Fuller	+	−	−	−	−	−

[a] Oligonucleotide probes: all = all species probe ITS 3; PH = all *Phytophthora* probe; pl = *P. palmivora* probe; cap = *P. capsici* probe; mg = *P. megakarya* probe; and cin = *P. cinnamomi* probe. The + indicates hybridization to the target DNA and the − indicates no hybridization. ND = no data.

[b] Isolates provided by M. Coffey at the University of California, Riverside, are preceded by p or c. Isolates provided by M. Dudzinsky at CSIRO, Canberra, Australia, are preceded by a. Isolates provided by R. Specker and M. S. Fuller at the University of Georgia, Athens, are preceded by GA. All other isolates were provided by B. Waalands at UC, Berkeley.

[c] DNA samples provided by R. Michelmore at UC, Davis.

with primers ITS 1 and ITS 4, beginning with 0.1–1.0 ng of total genomic DNA in an automated thermal cycler (Perkin-Elmer Cetus, Norwalk, CT), as described by White et al. (32). PCR amplifications containing no DNA template were carried out in every experiment as controls to test for the presence of contamination of reagents and reaction mixtures by nonsample DNA.

Probe design. Oligonucleotide probes (20 bp) that complemented taxon-specific ITS I sequences between the 185 and 5.85 ribosomal RNA genes were synthesized. These probes were identified from variable ITS sequences of *P. capsici, P. cinnamomi, P. megakarya,* and *P. palmivora* (21) and are listed in Table 2. The genus *Phytophthora* probe complemented a region of ITS I that was present in every *Phytophthora* isolate previously investigated (21), and a universally conserved probe (ITS 3) complemented a portion of the 5.85 rDNA, as described by White et al (32).

Probe preparation. Each probe was labeled with gamma $AT^{32}P$ phosphorylation at their 5′ ends, using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Kinase reactions consisted of 5 μl of probe (100 μM), 10 μl of 10× kinase buffer (0.7 M Tris-HCl, pH 7.6, 0.1 M $MgCl_2$-$6H_2O$, and 50 mM dithiothreitol), 5 μl of 100 mM $dAT^{32}P$ (3000 μCi per millimole), and 25 U of T4 kinase in 80 μl of water, as previously described (17,22). Kinase reactions were carried out for 30 min at 37 C, and the reactions were terminated by inactivating the enzyme at 68 C for 15 min.

TABLE 2. Nucleotide sequences and thermal features of oligonucleotide probes

Probe specificity	Nucleotide sequences	T_m[a]	T_h[b]	T_a[c]
All species (ITS 3)	5′-GCATCGATGAAGAACGCAGC	62	57	46
All *P.* species	5′-GCTATCTAGTTAAAAGCAGA	54	49	43
P. cinnamomi	5′-CAGTGATAGGGCCCGCCACG	68	63	64
P. palmivora	5′-GCAGCCGCCAGCAATGAAGC	66	61	61
P. megakarya	5′-GCAACCGCCAGTAATTAAAC	58	53	53
P. capsici	5′-TAATCAGTTTTGTGAAATGG	52	47	48

[a] T_m = temperature of melting.

[b] T_h = temperature of theoretical hybridization.

[c] T_c = temperature of actual hybridization that led to specificity.

Determination of probe hybridization temperatures for specificity. All hybridization temperatures that were used are listed in Table 2. The expected melting temperature (for probes smaller than 50 nucleotides) was calculated based on the probe-nucleotide

composition: $T_m = 4(G + C) + 2(A + T)$. The theoretical hybridization temperature (T_h) was calculated to be 5 C less than the T_m. The actual temperatures (T_a) that resulted in correct probe specificity also are listed in Table 2.

The T_m depends not only on the length and G + C content of the oligonucleotide, but also on the salt concentration of the hybridization buffer. Stringency of hybridization can be altered by either changing the salt concentration or the hybridization temperature ($T_m = 81.5C + 16.6_{\log}M + 0.41[\%G + \%C] - 500/n - 0.61[\%\text{formamide}]$, in which M is the ionic strength of the hybridization solution [moles per liter] and n is the length of the shortest chain in the duplex [5,8,24]).

Dot blot hybridization. Dot blots were prepared as described by Helmuth (14). In 100 μl of 0.4 N NaOH and 25 mM EDTA, 5 μl (50–100 ng) of amplified products were denatured for 5 min at room temperature. HYBOND N nylon membranes (Amersham, Arlington Heights, IL) were hydrated in H_2O for 1 min. After the membranes were placed in a dot blot apparatus (Hybri-Dot manifold, Bethesda Research Laboratories, Gaithersburg, MD), denatured samples (105 μl) were added to the appropriate wells and were slowly vacuum filtered onto the membrane. Afterward, 100 μl of TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) was filtered through each sample well. Nylon membranes were blotted dry with 3MM Whatman chromatography paper, and DNA was fixed with UV light, using 120 mJ per square centimeter at 254 nm (Stratalinker, Stratagene, La Jolla, CA). Replicate blots were prepared, one for each probe.

Nylon membranes spotted with DNA were prehybridized for 1 h at the hybridization temperature of the probes (Table 2) in 0.9 M NaCl; 0.05 M sodium phosphate; 5 mM EDTA; 0.2% SDS; 100 μg per milliliter of calf-thymus or salmon-sperm DNA; and 5× Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, and 1% BSA-Pentax Fraction V). A radioactive probe was added directly to this solution and was allowed to hybridize at either T_h or T_a for 1–2 h. Blots were washed three times for 15 min by agitating in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (23) and 0.1% SDS, at a wash temperature 5 C lower than the temperature of hybridization. Hybridized filters were used to expose X-ray film (Kodak X-AR) from 4 h to 1 day at −80 C. To reuse hybridized filters, radioactive probes were removed by two 15-min washes in boiling 1× SSC and 0.1% SDS.

RESULTS

Conditions for specific probe hybridization. In some experiments, increases above the calculated hybridization temperatures resulted in an increase in the probe specificity. For example, the T_a (the temperature of hybridization that resulted in specificity) was slightly higher than the T_h for both the *P. cinnamomi* and *P. capsici* probes (Table 2). For each of the four species probes, the T_a and T_h were identical or within 1 C. In other experiments, hybridization of the probes to the correct targets occurred at a range of temperatures below the theoretical values (ITS 3 the all species probe and the genus *Phytophthora* probe hybridized at 46–57 and 43–49 C, respectively).

Target DNA verification. DNA amplified by PCR with primers ITS 1 and ITS 4 was composed of both transcribed spacers ITS I and ITS II, as well as with the 5.85 rDNA. We used a universal probe, ITS 3, that was complementary to a conserved portion of 5.85 rDNA (32) to verify the presence of amplified target DNA on each hybridized filter (e.g., Fig. 1, lane 1; Table 1). Target DNA from all isolates except *P. citrophthora* (Fig. 1, row d) was present in nearly equal amounts (Fig. 1, lane 1).

Discrimination of *Phytophthora* from other Oomycete genera. The genus *Phytophthora* probe, designed to complement an ITS sequence shared by every tested species of *Phytophthora*, hybridized only to the target DNA of *Phytophthora* species, not to that of 12 other genera (Table 1). For example, the genus *Phytophthora* probe hybridized to target DNA of *P. palmivora, P. capsici, P. megakarya, P. cinnamomi* (Al- and A2-mating type), and *P. citrophthora* isolates (Fig. 1, lane 2, rows a–f) but not to the amplified targets of *Pythium oligandrum* or *Peronospora parasitica* (Fig. 1, lane 2, rows g–h). The genus *Phytophthora* probe also hybridized to other *Phytophthora* species isolates, which included *P. cactorum, P. megasperma,* and *P. gonapodyides* (Table 1).

Detection of *Phytophthora* species using oligonucleotide probes. Each of the following species probes hybridized only to their respective targets and not to the target DNA of other *Phytophthora* species nor to the other Oomycete genera.

The oligonucleotide probe based on the *P. cinnamomi* sequence hybridized only to the target DNA of *P. cinnamomi* (Fig. 1, lane 6, rows e and f). This probe distinguished 18 *P. cinnamomi* isolates from all other species tested (Table 1). The target DNA of one unusual *P. cinnamomi* isolate, 2264, did not hybridize with the *P. cinnamomi* probe (data not shown). Isolate 2264 also had a different ITS II sequence (21), different mitochondrial DNA restriction-fragment patterns (S. B. Lee, *unpublished data*), and an unusual isozyme profile, compared to other *P. cinnamomi* isolates examined (P. Oudemans, *personal communication*). This evidence is consistent with the idea that isolate 2264 may not be classified correctly (21). Specificity of hybridization occurred at 64 C.

The oligonucleotide probe based on the *P. palmivora* sequence hybridized strongly only to the target DNA of the two *P. palmivora* isolates tested (Table 1). Specificity of hybridization occurred at 61 C (Fig. 1, lane 3).

The oligonucleotide probe based on the *P. capsici* sequence hybridized strongly only to the target DNA of the two *P. capsici* isolates tested (Table 1). Specificity of hybridization occurred at 48 C (Fig. 1, lane 4).

The oligonucleotide probe based on the *P. megakarya* sequence hybridized strongly only to its target DNA, not to any other isolates of *Phytophthora* nor to any other outgroup isolates tested (Table 1). Specificity of hybridization occurred at 53 C (Fig. 1, lane 5).

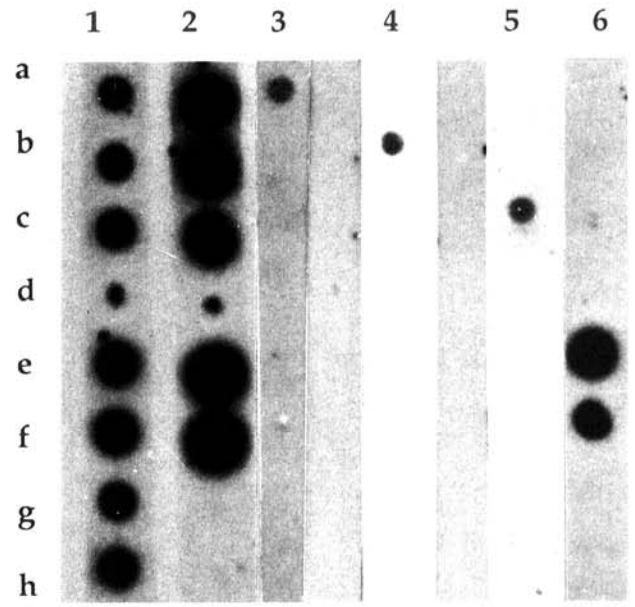

Fig. 1. DNA-DNA hybridization between nylon membrane-bound target DNA and radiolabeled probe DNA. Target DNA was PCR amplified, ITS spotted on nylon membranes from (top to bottom) *P. palmivira* (255), *P. capsici* (622), *P. megakarya* (1664), *P. citrophthora* (449), *P. cinnamomi* (2100), *P. cinnamomi* (2110), *Pythium oligandrum* (51-020), and *Peronospora parasitica* (rows a–h, respectively). Numbers in parentheses refer to isolates. Each radiolabeled DNA probe was used to hybridize one replicate blot. Lane 1, all species probe ITS 3; lane 2, all *Phytophthora* species probe; lane 3, *P. palmivora* species-specific oligonucleotide; lane 4, *P. capsici* species-specific oligonucleotide; lane 5, *P. megakarya* species-specific oligonucleotide; and lane 6, *P. cinnamomi* species-specific oligonucleotide.

DISCUSSION

Ribosomal DNA ITS species-specific oligonucleotides developed for *P. cinnamomi, P. palmivora, P. megakarya,* and *P. capsici* provide new tools for rapid species identification in *Phytophthora*. In addition, a genus *Phytophthora* probe distinguished *Phytophthora* species from representatives of 12 other Oomycete genera.

Our experiments tested only a limited number of isolates from each species. The largest sampling was 18 *P. cinnamomi* isolates. Screening more isolates from each species is necessary for a better evaluation of the specificity and the range of the oligonucleotide probes. The speed of amplification and dot blot hybridization assay is well suited to the analysis of many isolates from each of the *Phytophthora* species.

Other molecular methods have been used to delineate species of *Phytophthora*. Repetitive DNA polymorphism analysis has been used to distinguish 12 *Phytophthora* species (26). Species-specific cloned repetitive-DNA fragments also have been used to identify isolates of *P. parasitica* and to detect *P. citrophthora* from cultures as well as soil and host tissue (11–13). Although these methods are useful, they are limited for several reasons. Nanogram amounts of DNA for every test are necessary for detection by cloned DNA probes. Repetitive DNA polymorphism analysis also requires large amounts of DNA (5 μg) per test. Furthermore, the potential loss of target sequences resulting from recombination and deletion events may limit the use of these approaches because the target DNA sites are unknown.

PCR-based DNA methods have also been used to detect other plant-pathogenic fungi, including *Gaeumannomyces graminis* (16) and *Phoma tracheiphila* (28). In the mycorhizzal basidiomycete genus *Laccaria*, PCR-based identification involving ribosomal DNA internal transcribed spacer probes was used to distinguish different strains of one species, *Laccaria bicolor* (10). In addition, studies involving rDNA nontranscribed spacer probes previously identified the yeast, *Metschnikowia reukaufii* (15).

Use of PCR-based identification has many advantages over use of traditional taxonomy and other molecular-identification schemes. PCR is primer directed, and as such, the primers can be designed to specifically amplify fungal DNA from heterogenous plant-fungal samples such as mycorrhizae (10). This obviates the need to extract pure fungal cultures from infected plant tissue. PCR can be performed on very small biological samples (i.e., single spores [20]) or herbarium-preserved fungi (6) and can be used to analyze unculturable, obligate plant parasites, such as *Albugo candida* (this study).

Because of the specificity of oligonucleotide probes and the sensitivity of PCR, this method is capable of detecting the presence of a 1 bp difference (10), in as little as one molecule of target DNA (22). Lee and Taylor anticipate that detecting variability in single spores of *Phytophthora* will be useful for investigating segregation of molecular markers after mating (29). The addition of nonradioactive methods of detection (14) to the battery of *Phytophthora* species-specific oligonucleotides will make the methods safer and easier to use. The high level of sensitivity may be especially useful in screening for *Phytophthora* infestations of soil and plant products, in research, in quarantine, and for control purposes. The use of PCR and DNA probes may provide sensitive tools that can be useful aides in rapid, reliable identification of *Phytophthora* species and that can be especially useful for disease diagnosis and for research in ecology and epidemiology.

As the catalog of *Phytophthora* rDNA ITS-sequence data is extended, it should be possible to apply this technique to other *Phytophthora* species and to ultimately provide probes for each species.

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