Identification of Some Oomycetes by Reverse Dot Blot Hybridization

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ABSTRACT

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An assay was developed that can identify unknown isolates of *Pythium* or *Phytophthora* species in a single hybridization. This reverse dot blot system is based on arrays of species-specific amplified fragments or oligonucleotides derived from the internal transcribed spacer (ITS) region, which are blotted as dots on a nylon membrane. By using total DNA from a sample as the template, universal primers, and digoxigenin-dUTP, the ITS was amplified and labeled simultaneously by the polymerase chain reaction (PCR). A small aliquot of the resultant labeled and amplified product was used as a probe for hybridization to a dot blot membrane that contained the immobilized species-specific oligonucleotides or amplified PCR fragments. The reverse dot blot system based on arrays of oligonucleotides showed far fewer cross-hybridizations than one based on entire amplified ITS I fragments. Unknown species can be identified simply by visualizing the positive hybridization reaction between the DNA labeled directly from the sample and the immobilized specific oligonucleotide. Currently, the assay can be used to identify *Pythium aphanidermatum*, *P. ultimum*, *P. acanthicum*, and *Phytophthora cinnamomi*. An oligonucleotide that was originally designed to identify *Phytophthora* hybridized to 10 of the 14 *Phytophthora* species tested. Another oligonucleotide designed to identify oomycetes hybridized to the 68 species tested, which represented two of the four orders of this phylum.

Additional keywords: chemiluminescence, detection, diagnosis, NIH IMAGE.

Identification of oomycetes is still based primarily on morphological characters (6,40). High variability within key structures leads to considerable overlap between species and to potential errors in identification, especially for mycologists who do not have many years of experience working with this group (13,14). Molecular techniques can be useful not only to identify species, but to better understand the evolutionary relationships between species (7,25). Separation of proteins by electrophoresis has been used to differentiate *Pythium* species (4,9), and a species-specific antibody for *P. ultimum* has been developed (44). Restriction fragment length polymorphism (RFLP) of total DNA (29) or of amplified ribosomal DNA (8,10) has been used to differentiate and study relationships between certain *Pythium* species. Spacers of the genomic ribosomal DNA cistron amplified by polymerase chain reaction (PCR) have been used as species-specific probes in standard dot blot assays (23,30). For some species of *Phytophthora*, oligonucleotide probes were designed by comparing their internal transcribed spacer (ITS) sequences (26,27), and species-specific genomic DNA probes selected from random clones have been developed for *P. citrophthora* and *P. nicotianae* (synanamorph *P. parasitica*) (16,17).

These techniques, particularly the standard dot blot and antibody-based assays, are convenient to test many samples for the presence of one particular pathogen. However, they are not efficient to identify an isolate when there are many possibilities to be addressed (e.g., when isolates lack reproductive structures) or when several different pathogens that might be present within a given sample are to be tested simultaneously. There are some recent studies in which a few pathogens were assayed simultaneously. For example, two species-specific pairs of primers were simultaneously used in a single PCR reaction to test for the presence of two pathogens (3,18). An interesting variation of this technique was developed by using a fluorescent dye-labeled nested primer located between a pair of specific primers (38). This technology, called TaqMan, can potentially detect up to three pathogens in a single reaction without having to visualize the results on an agarose gel. Such multiplex PCR reactions are limited by the number of primer pairs that can be used in a single reaction, the number of bands that can be clearly identified to a species level without giving false positives, and/or the number of different fluorescent dyes currently available. There is therefore a need for an approach in the molecular identification of fungi that could detect large numbers of species at once.

A relatively novel technique called the reverse dot blot has been successfully used to detect, in a single assay, a range of mutations related to different human disorders (36) and more recently to monitor bacteria from environmental samples (41). This technique involves the use of multiplex PCR to simultaneously amplify and label the regions of the DNA that are used to design the specific oligonucleotides. The labeled PCR products are used as probes for hybridization with a membrane that contains an array of specific oligonucleotides. Mutations or species present in a sample are determined from the positive reactions in the hybridization test. Reverse dot blot has also been used with specific PCR fragments blotted on the membrane and labeled genomic DNA used for probing (24). In a similar fashion, it was used with a single dot to detect *Erwinia amylovora* (33).

The objective of this study was to demonstrate the feasibility of the reverse dot blot technique for the identification of selected oomycetes: *Pythium aphanidermatum*, *P. acanthicum*, *P. ultimum*, and *Phytophthora cinnamomi*. Identification at the phylum level for the oomycetes and at the genus level for *Phytophthora* was also undertaken.

MATERIALS AND METHODS

Fungal collection. A collection of 166 isolates was used in this study (Table 1). Cultures of oomycetes were maintained on slants

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TABLE 1. Isolates used for various parts of this study

Phylum	Order	Species	Culture[a]	Origin	Host or substrate	Fig. 1[b]	Figs. 3–6[c]	RDBH tested[d]
Ascomycota	Diaporthales	*Diaporthe vaccinii*	FLC 93014B	MA	*Vaccinium macrocarpon*		x	x
	Eurotiales	*Penicillium lividum*	NRRL754	NA[e]	NA		x	x
	Unknown	*Allantopomopsis lycopodina*	FLC 85195	MA, U.S.A.	*Vaccinium macrocarpon*			x
Chytridiomycota	Blastocladiellales	*Allomyces macrogynus*	ATCC 38327	NA	Pond soil		x	x
		Blastocladiella emersonii	ATCC 22665	PA, U.S.A.	Pond water		x	x
	Spizellomycetales	*Olpidium bornovanus*	DMR	NA	*Cucumis sativus*		x	x
Oomycota	Saprolegniales	*Saprolegnia ferax*	ATCC 36051	Canada	Lake water		x	x
		Aphanomyces euteiches	ATCC 46690	WI, U.S.A.	*Pisum sativum*		x	x
		Dictyuchus monosporus	ATCC 34931	TN, U.S.A.	Lake water		x	x
		Achlya recurva	ATCC 16088	MD, U.S.A.	Soil		x	x
		Achlya sp.	CAL K2	B.C., Canada	Hydroponic solution		x	x
	Peronosporales	*Peronophythora litchii*	ATCC 28739	Taiwan	*Litchi chinensis*		x	x
		Pythium acanthicum	CBS 377.34	Sweden	*Solanum tuberosum*	x	x	x
		P. acanthicum	DJSB 500	Ont., Canada	Soil			x
		P. acanthicum	ZGA	NC	Turfgrass			x
		P. acanthicum	DJSB 178	Ont., Canada	*Hordeum vulgare*			x
		P. acanthicum	FNM 66-1	FL, U.S.A.	Soil			x
		P. acanthicum	FNM A-6	NC, U.S.A.	Soil			x
		P. acanthophoron	DJSB 496	B.C., Canada	*Malus pumila*		x	x
		P. afertile	DJSB 417	Alb., Canada	*Triticum aestivum*		x	x
		P. aphanidermatum	DJSB 444	B.C., Canada	*Cucumis sativus*			x
		P. aphanidermatum	CBS 118.80	France	NA	x		x
		P. aphanidermatum	DJSB 206	Ont., Canada	*Pinus resinosa*			x
		P. aphanidermatum	ZGA	NC, U.S.A.	Turfgrass			x
		P. aphanidermatum	DJSB 206	Ont., Canada	*Pinus resinosa*			x
		P. aphanidermatum	DJSB 740	B.C., Canada	*Cucumis sativus*			x
		P. aphanidermatum	JM 100	B.C., Canada	*Cucumis sativus*		x	x
		P. aphanidermatum	FNM 96-4	FL, U.S.A.	Soil			x
		P. aphanidermatum	FNM 1992-31	South Africa	NA			x
		P. aphanidermatum	FNM 1991-3	Japan	NA			x
		P. aphanidermatum	FNM 1987-61	Mexico	*Lactuca sativa*			x
		P. aphanidermatum	WC 25-2	NA	NA			x
		P. aphanidermatun	WC 21-4	NA	NA			x
		P. aquatile	CAL 1695	B.C., Canada	Hydroponic solution		x	x
		P. aristosporum	ZGA	NC, U.S.A.	Turfgrass			x
		P. aristosporum	CBS 263.38	Sask., Canada	*Triticum aestivum*		x	x
		P. arrhenomanes	DJSB 671	Man., Canada	*Avena fatua*	x		x
		P. arrhenomanes	CBS 324.62	WI, U.S.A.	*Zea mays*		x	x
		P. carolinianum	ZGA	NC, U.S.A.	Turfgrass		x	x
		P. catenulatum	CBS 842.68	SC, U.S.A.	Turfgrass		x	x
		P. coloratum	DJSB 401	NA	NA	x		x
		P. coloratum	CBS 154.64	S. Australia	Soil		x	x
		P. deliense	CBS 114.84	Peru	*Solanum tuberosum*			x
		P. deliense	CBS 314.33	Indonesia	*Nicotiana tabacum*		x	x
		P. dissotocum	CBS 166.68	OH, U.S.A.	*Triticum aestivum*	x	x	x
		P. echinulatum	DJSB 327	Australia	Grass		x	x
		P. flevoense	CBS 234.72	Netherlands	Soil		x	x
		P. graminicola	CBS 327.62	Jamaica	*Saccharum officinarum*		x	x
		Pythium group F	RD 55	B.C., Canada	*Phaseolus vulgaris*		x	x
		Pythium group F	RD 49	B.C., Canada	*Phaseolus vulgaris*			x
		Pythium group F	RD	NA	NA			x
		Pythium group G	CAL 1478	B.C., Canada	*Phaseolus vulgaris*		x	x
		Pythium group G	RD 37	B.C., Canada	Soil	x		x
		Pythium group G	RD 51	B.C., Canada	Soil			x
		P. helicoides	CBS 286.31	U.S.A.	*Phaseolus vulgaris*		x	x
		P. heterothallicum	CBS 450.67	Canada	*Sambucus* sp.	x		x
		P. heterothallicum	CBS 451.67	Canada	*Sambucus* sp.	x	x	x
		P. hypogynum	IMI 242092	U.K.	*Petroselinum crispum*			x
		P. hypogynum	DJSB 393	Qué., Canada	*Chenopodium album*	x		x
		P. intermedium	CAL 1217	AK, U.S.A.	Water baiting	x	x	x
		P. intermedium	CBS 266.38	Netherlands	*Agrostis* sp.			x
		P. irregulare	CBS 250.28	Netherlands	*Phaseolus vulgaris*	x	x	x
		P. iwayamai	CBS 156.64	Australia	*Pinus* sp.	x		x
		P. iwayamai	CBS 697.83	Japan	*Triticum* sp.		x	x
		P. macrosporum	DJSB 492	Netherlands	Flower bulb		x	x
		P. mamillatum	DJSB 324	India	*Carthamus tinctorius*			x
		P. mamillatum	CBS 251.28	Netherlands	*Beta vulgaris*			x
		P. mamillatum	DJSB 326	Australia	*Malus pumila*			x
		P. mamillatum	DJSB 765	B.C., Canada	Cucumber		x	x
		P. multisporum	CBS 470.50	U.S.A.	Soil	x	x	x
		P. myriotylum	CBS 254.70	Israel	*Arachis hypogaea*		x	x
		P. okanoganense	CBS 315.81	U.S.A.	*Triticum aestivum*	x	x	x
		P. oligandrum	CBS 382.34	U.K.	*Viola* sp.	x	x	x
		P. oligandrum	ZGA	NC, U.S.A.	Turfgrass			x
		P. oligandrum	DJSB 262	Ont., Canada	*Pisum sativum*			x

(*continued on next page*)

[a] ATCC = American Type Culture Collection, Rockville, MD; BCMAFF = from collection of B. C. Ministry of Agriculture, Fisheries and Food, Abbotsford, B.C., Canada; CAL = from collection of C. A. Lévesque; CBS = Centraalbureau voor Schimmelcultures, Baarn, Netherlands; DJSB = from collection of Donald J. S. Barr, Eastern Cereal and Oilseed Research Centre, Ottawa, Ontario, Canada; DMR = DNA provided by D. M. Rochon, Agriculture and Agri-Food Canada, Summerland, B.C., Canada; FLC = from collection of F. L. Caruso, Cranberry Experimental Station, East Wareham, MA; FNM = from collection of F. N. Martin, USDA-ARS, Salinas, CA; IMI = International Mycological Institute, Surrey, England; MJD = from collection of M. J. Drilias, University of Wisconsin, WI; NRRL = Northern Regional Research Laboratory, USDA, Peoria, IL; PBH = from collection of P. B. Hamm, Oregon State University, Hermiston, OR; RD = from collection of Rolando Descalzo, IG MicroMed Environmentals, Richmond, B.C., Canada; WC = from collection of W. Chen, Illinois Natural History Survey, Champaign, IL; ZGA = strains provided for *Pythium* identification workshop organized by Z. G. Abad, North Carolina State University, Raleigh.

[b] Isolates that had their amplification products blotted on membranes shown in Figure 1.

[c] Isolates used for hybridization that are shown in Figures 3–6.

[d] Isolates tested by reverse dot blot hybridization with oligonucleotides.

[e] Information not available.

TABLE 1. (*continued from preceding page*)

Phylum	Order	Species	Culture[a]	Origin	Host or substrate	Fig. 1[b]	Figs. 3–6[c]	RDBH tested[d]
		P. paroecandrum	CBS 157.64	Australia	Soil	x	x	x
		P. periilum	ZGA	NC, U.S.A.	Turfgrass		x	x
		P. pleroticum	DJSB 257	Ont., Canada	*Pisum sativum*		x	x
		P. polymastum	DJSB 494	Netherlands	*Lectuca sativa*		x	x
		P. pulchrum	ZGA	NC, U.S.A.	Turfgrass		x	x
		P. pyrilobum	CBS 158.64	Australia	*Pinus radiata*	x	x	x
		P. rostratum	CBS 172.68	U.K.	*Medicago sativa*	x		x
		P. rostratum	CBS 533.74	Netherlands	Soil		x	x
		P. salpingophorum	DJSB 627	Alb., Canada	*Brassica napus*			x
		P. salpingophorum	CBS 471.50	Germany	*Lupinus* sp.			x
		P. salpingophorum	DJSB 897	B.C., Canada	Turfgrass			x
		P. salpingophorum	DJSB 887	Man., Canada	*Triticum aestivum*			x
		Pythium unknown	BCMAFF 93-224A	B.C., Canada	*Rubus idaeus*			x
		Pythium sp.	CAL 1493	B.C., Canada	*Vaccinium macrocarpon*			x
		Pythium sp.	CAL 1495	B.C., Canada	*Vaccinium macrocarpon*			x
		Pythium sp.	CAL 1497	B.C., Canada	*Vaccinium macrocarpon*			x
		Pythium sp.	CAL 1499	B.C., Canada	*Vaccinium macrocarpon*			x
		P. spinosum	ZGA	NC, U.S.A.	Turfgrass		x	x
		P. spinosum	CBS 275.67	Netherlands	Compost			x
		P. splendens	DJSB 435	NJ, U.S.A.	*Vaccinium corymbosum*	x		x
		P. splendens	CBS 462.48	U.S.A.	NA		x	x
		P. sulcatum	DJSB 195	B.C., Canada	*Daucus carota*	x	x	x
		P. sulcatum	CBS 603.73	WI, U.S.A.	*Daucus carota*		x	x
		P. sylvaticum male	CAL 1446	B.C., Canada	*Triticum aestivum*	x		x
		P. sylvaticum female	CAL 1476	B.C., Canada	*Phaseolus vulgaris*	x		x
		P. sylvaticum female	CAL 1498	B.C., Canada	*Vaccinium macrocarpon*			x
		P. sylvaticum	CBS 453.67	TN, U.S.A.	Soil		x	x
		P. tardicrescens	ZGA	NC, U.S.A.	Turfgrass		x	x
		P. torulosum	CBS 316.33	Netherlands	Grass root		x	x
		P. ultimum var. *ultimum*	CBS 398.51	Netherlands	Lepidum sativum	x		
		P. ultimum var. *ultimum*	IMI 334959	Peru	*Solanum tuberosum*			x
		P. ultimum var. *ultimum*	CBS 291.31	NA	*Dioscorea batatas*			x
		P. ultimum var. *ultimum*	CBS 296.37	U.K.	*Pisum sativum*		x	x
		P. ultimum var. *ultimum*	CBS 488.86	Poland	*Malus sylvestris*			x
		P. ultimum var. *ultimum*	CAL 1444	B.C., Canada	*Phaseolus vulgaris*			x
		P. ultimum var. *ultimum*	CAL 1447	B.C., Canada	*Triticum aestivum*			x
		P. ultimum var. *ultimum*	CAL 1491	B.C., Canada	*Vaccinium macrocarpon*			x
		P. ultimum var. *ultimum*	CAL 1492	B.C., Canada	*Vaccinium macrocarpon*			x
		P. ultimum var. *ultimum*	CAL 1494	B.C., Canada	*Vaccinium macrocarpon*			x
		P. ultimum var. *sporangiiferum*	CBS 219.65	U.S.A.	*Chenopodium album*	x		x
		P. ultimum var. *sporangiiferum*	CBS 171.68	U.K.	*Medicago sativa*		x	x
		P. ultimum var. *sporangiiferum*	CBS 111.65	Lebanon	*Medicago sativa*			x
		P. ultimum	RD	B.C., Canada	Soil			x
		P. undulatum	CAL 1232	AK, U.S.A.	NA	x		x
		P. undulatum	CBS 157.69	AL, U.S.A.	Soil		x	x
		P. vanterpoolii	DJSB 159	Ont., Canada	*Triticum aestivum*		x	x
		P. vexans	CBS 119.80	Iran	Soil			x
		P. violae	CBS 159.64	Australia	Soil	x		x
		P. violae	CBS 178.86	Netherlands	*Daucus carota*	x		
		P. violae	CBS 159.64	S. Australia	Soil		x	x
		P. volutum	ZGA	NC, U.S.A.	Turfgrass		x	x
Oomycota	Peronosporales	*Phytophthora cactorum*	CAL 842	U.K.	NA			x
		P. cactorum	DJSB 153	N. S., Canada	*Fragaria* sp.		x	x
		P. cambivora	CAL 841	U.K.	*Rubus idaeus*		x	x
		P. cambivora	PBH 150	OR, U.S.A.	*Abies* sp.			x
		P. cinnamomi	FLC 9216	MA, U.S.A.	*Vaccinium macrocarpon*			x
		P. cinnamomi	FLC 9217	MA, U.S.A.	*Vaccinium macrocarpon*		x	x
		P. cinnamomi A2	DJSB 132	U.K.	*Erica* sp.			x
		P. cinnamomi	MJD W.021	MA, U.S.A.	*Vaccinium macrocarpon*			x
		P. cinnamomi	MJD W.244	OR, U.S.A.	*Vaccinium macrocarpon*			x
		P. cinnamomi	MJD W.253	NJ, U.S.A.	*Vaccinium macrocarpon*			x
		P. cinnamomi A2	BCMAFF 94-1161	B.C., Canada	*Vaccinium corymbosum*			x
		P. citricola	PBH 232	MN, U.S.A.	*Abies* sp.		x	x
		P. cryptogea	PBH 8819	MA, U.S.A.	*Vaccinium macrocarpon*			x
		P. cryptogea	PBH 8823	MA, U.S.A.	*Vaccinium macrocarpon*			x
		P. cryptogea	CBS 113.19	NA	*Lycopersicon esculentum*			x
		P. cryptogea	MJD W.090	WI, U.S.A.	*Vaccinium macrocarpon*			x
		P. cryptogea	MJD W.234	MA, U.S.A.	*Vaccinium macrocarpon*			x
		P. cryptogea	MJD W.240	OR, U.S.A.	*Vaccinium macrocarpon*		x	x
		P. drechsleri	PBH 133	B.C., Canada	*Pseudotsuga menziensii*		x	x
		P. erythroseptica	PBH 577	OR, U.S.A.	*Solanum tuberosum*		x	x
		P. fragariae race A4	CAL 1414	N. S., Canada	*Fragaria* sp.			x
		P. fragariae var. *rubi*	CAL 1417	U.K.	*Rubus idaeus*		x	x
		P. infestans A2	CAL 1424	Canada	*Solanum tuberosum*			x
		P. infestans A1	CAL 1426	Canada	*Solanum tuberosum*		x	x
		P. infestans A1	CAL 1428	Switzerland	*Solanum tuberosum*			x
		P. inflata	PBH 351	NA	NA		x	x
		P. lateralis	PBH 632	OR, U.S.A.	*Chamaecyparis lawsoniana*			x
		P. lateralis	PBH 366	CA, U.S.A.	*Chamaecyparis lawsoniana*			x
		P. lateralis	PBH 367	NA/	*Chamaecyparis lawsoniana*			x
		P. megasperma	FLC 8806	MA, U.S.A.	*Vaccinium macrocarpon*		x	x
		P. megasperma	FLC 8815	MA, U.S.A.	*Vaccinium macrocarpon*			x
		P. megasperma	MJD W.088	WI, U.S.A.	*Vaccinium macrocarpon*			x
		P. nicotianae	DJSB 235	Ont., Canada	*Poinsettia* sp.		x	x
		P. syringae	PBH 210	NY, U.S.A.	*Malus pumila*		x	x
Zygomycota	Mucorales	*Mortierella alpina*	DAOM 175051	Alb., Canada	Soil		x	x
		Mortierella sp.	BCMAFF 94-1148	B.C., Canada	*Vaccinium macrocarpon*			x
		Mortierella sp.	BCMAFF 94-1151	B.C., Canada	*Vaccinium macrocarpon*			x
		Phycomyces nitens	ATCC 9984	NA	NA		x	x

under mineral oil and in water (11). This collection contains several genera of Oomycetes and a few of Chytridiomycetes, Zygomycetes, and Ascomycetes.

Fungal growth and DNA extraction. All isolates were grown according to previously published procedures (30). DNA was extracted from 5- to 10-day-old cultures as described previously (19). DNA pellets were resuspended in Tris-EDTA and diluted to a concentration of approximately 50 ng μl^{-1}.

DNA amplification and labeling. For preparing membranes with immobilized ITS I region, approximately 10 ng of total DNA template was amplified by PCR with primers ITS1 and ITS2 (43). The PCR reagents were obtained from a kit (Perkin Elmer, Norwalk, CT). A DNA thermocycler 480 (Perkin Elmer) was used for 39 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C with a final 10-min extension at 72°C. For labeling, conditions were as described above, except that primers ITS1 and ITS4 were used with 16.7 µM dTTP and 8.3 µM alkaline labile DIG-dUTP (digoxigenin-11-dUTP; Boehringer Mannheim GmbH, Mannheim, Germany) instead of 200 µM dTTP. The final product was ethanol-precipitated in LiCl according to the protocol from the manufacturer of DIG-dUTP.

Blotting entire ITS I. PCR products were gel purified with the Wizard PCR preps kit (Promega, Madison, WI) and quantified by using λ DNA digested with *Cla*I to provide a size standard at 2.2 ng μl^{-1} for the smallest band of 290 bp, the average size of the PCR products obtained. The gels were photographed over a 300-nm UV light source. Densitometry of a scanned negative of the gel with the software NIH IMAGE (35) was done to generate a standard curve, to estimate the concentration of gel-purified PCR products, and to adjust to a final concentration of 12.5 ng μl^{-1}. An aliquot of 2 µl of DNA was mixed with 0.5 µl of 2.5 N NaOH, and 0.2 µl of the mix was dotted (2 ng per dot) 6 mm apart on a dry, positively charged nylon membrane (Boehringer). Membranes were baked at 120°C for 1 h.

Hybridization with immobilized PCR products. Membranes were prehybridized in 20 ml of hybridization solution (Boehringer) for a minimum of 1 h at one of the hybridization temperatures listed below. Between 2 and 10 ng of DNA from the PCR reaction product was used as a probe for hybridization in 8-ml volume. Overnight hybridization, a room-temperature wash in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), and two final washes in 0.1× SSC were done according to the procedure of Boehringer. Hybridizations and final washes were performed at 60 and 68°C, 60 and 73°C, 68 and 68°C, 68 and 73°C, 68 and 80°C, 68 and 95°C, 72 and 72°C, 72 and 76°C, 75 and 75°C, 75 and 80°C, 75 and 95°C, 80 and 80°C, and 80 and 95°C, respectively. Digoxigenin was detected by chemiluminescence according to the protocol from the manufacturer by using 3-(2′spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane (Lumigen-PPD) with 15- to 60-min film exposure (X-Omatic K; Kodak, Rochester, NY) on the day after the Lumigen-PPD was added. Hybridizations were done at least twice. Membranes were erased according to the procedure of Boehringer for alkaline-labile DIG-dUTP.

TABLE 2. Nucleotide sequences of oligonucleotides used for reverse dot blot

Specificity	Nucleotide sequence
Oomycete	5′-TGC GGA AGG ATC ATT ACC ACA C
Phytophthora spp.	5′-TTC CAC GTG AAC CGT ATC AAC
Pythium acanthicum-92	5′-AAG ATT TGA GGC TGA ACG AA
Pythium acanthicum-132	5′-GAT GCG GAT TTG CTG ATG TTA
Pythium aphanidermatum	5′-TTT TGG AGT ATA GAT CAG TAT TAG GTA AA
Pythium ultimum	5′-TGC TGA CTC CCG TTC CAG TG
Phytophthora cinnamomi[a]	5′-CGT GGC GGG CCC TAT CAC TG
Positive control (ITS2)[b]	5′-GCT GCG TTC TTC ATC GAT GC
Positive control (ITS4)[b]	5′-TCC TCC GCT TAT TGA TAT GC

[a] Forward version of primer from Lee and Taylor (27).
[b] From White et al. (43).

DNA sequencing and oligonucleotide selection. Sequencing of ITS I for *P. aphanidermatum* (DJSB 444), *P. ultimum* (CBS 398.51), and *P. acanthicum* (CBS 377.34) isolates was performed with the AmpliTaq Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and a 373 automated sequencer

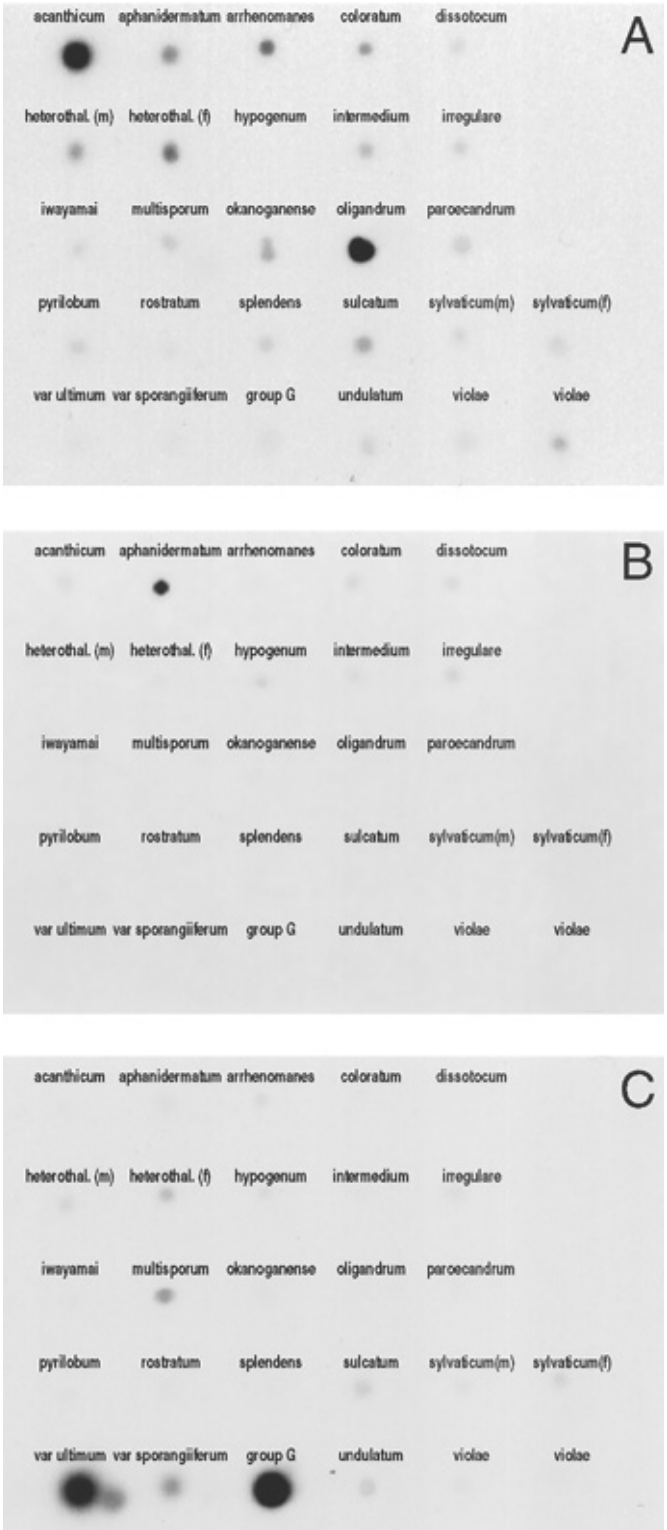

Fig. 1. Reverse dot blot hybridizations in which polymerase chain reaction (PCR)-amplified internal transcribed spacers (ITS) were blotted directly onto the nylon membranes. The isolates used to produce the blotted PCR products are identified in Table 1. PCR-amplified and labeled ITS regions of the following species were used as probes: **A,** *Pythium acanthicum*, CBS 377.34; **B,** *P. aphanidermatum*, CBS 118.80; and **C,** *P. ultimum* var. *ultimum*, CBS 398.51.

from the same manufacturer. Templates, prepared by PCR as described above with primers ITS1 and ITS4 (43), were purified with NucleotrapPCR (Macherey-Nagel, Düren, Germany) and used at 250 ng per sequencing reaction with 3.2 pmol of primers ITS1 or ITS 2 (43). *Pythium* and *Phytophthora* (26) sequences were aligned with GCG 7.0 (Genetic Computer Group, Madison, WI), and the software Oligo 4.0 (National Biosciences, Plymouth, MN) was used in the design of the oligonucleotides.

Blotting oligonucleotides. Oligonucleotides listed in Table 2 were poly(dT)-tailed at the 3′ end and blotted according to the procedure of Kawasaki et al. (22). A control with the ITS I and ITS II regions amplified with the primer pair ITS5 and ITS4 (43) was also added by using 4 ng total of an equal mix of amplified DNA of *Gloeosporium* sp., *Phytophthora megasperma*, *Pythium heterothallicum*, and *Mortierella* sp. The detection control dot contained 2 ng of the same ITS region of *Pythium ultimum* that had been labeled by PCR with alkaline stable DIG-dUTP. These controls were blotted after heat denaturation. Membranes were irradiated by UV (Stratalinker 1800; Stratagene, San Diego, CA) to bind the DNA (22).

Hybridization with immobilized oligonucleotides. Membranes were prehybridized in 20 ml of hybridization solution (Boehringer) for a minimum of 1 h at the hybridization temperature described below. From 50 to 100 ng of labeled DNA from the PCR reaction was added to 8 ml of hybridization buffer. Hybridization for a minimum of 2 h, a room temperature wash in 2× SSC, and two final washes were done according to the procedure of Boehringer. A subset of isolates was used, and hybridizations and washes were done at 55, 58, 60, and 65°C in addition to hybridization and final wash combinations of 45 and 58°C and 55 and 58°C, respectively. Final washes were done subsequently in either 0.1, 0.5, 1.0, or 2.0× SSC. Digoxigenin was detected as stated above. Membranes were erased according to the procedure of Boehringer for alkaline labile DIG-dUTP.

RESULTS

Reverse dot blot with immobilized PCR products. Differences among patterns of hybridization dots were obtained for a range of *Pythium* species, but identification to species level was difficult. Even under conditions of very high stringency, background and cross-hybridization reactions were observed. For example, hybridization at 80°C of reverse dot blot membranes with *P. acanthicum* DNA followed by washes at the same temperature with 0.1× SSC gave extensive cross-hybridization (Fig. 1A). Other species, such as *P. irregulare*, exhibited an even higher level of cross-hybridization (data not shown). Fungi such as *P. aphanidermatum* (Fig. 1B) and *P. ultimum* (Fig. 1C) that hybridized under the same conditions showed marginally lower levels of cross-hybridization. Similar results were observed when this approach was used with some *Phytophthora* species (data not shown).

Sequencing and oligonucleotide design. Sequences of the ITS I regions were determined for three different *Pythium* species (Fig. 2). Potential species-specific, genus-specific, and oomycete-specific primers were designed by using this alignment and that of Lee and Taylor (26). Selected oligonucleotides were tested by reverse dot blot (Table 2).

Reverse dot blots with immobilized oligonucleotides. The optimum conditions selected for hybridizations and washes were determined to be 55°C followed by final washes with 2.0× SSC. The oomycete oligonucleotide hybridized to seven different genera

```
Consensus   GTGAACCTGC GGAAGGATCA TTACCACACY WWAAAAAACT KTCCACGTGA      50
ultimum     .......... .......... .........T TT........ G.........      50
aphanider.  .......... .......... .........C AT.....-.. T.........      49
acanthicum  .......... .......... .........C TA....--.. T.........      48

Consensus   ACYGTAWRHA AGTYYAGCGC TGTGWCTGWG CYGGTGTYKT CATTKTYGGA     100
ultimum     ..T...AGC. ...CT..... ....A...A. .T.....TT. ....T.T...     100
aphanider.  ..C..-TGA. .--TC.---- ...-T...T. .------.C-. .-..-.C-.-     81
acanthicum  ..C..-TAT. .--CT.---- ...-T...T. .C----.CG. .-..G.T-..     84

Consensus   CACTRGAAYK KGAGTCAGCW GRACGAAGGT GGGCTGCTTA ATTGTAGTCT     150
ultimum     ....G...CG G........A .G........ ---------- --..--....     136
aphanider.  ----G.-.-- -.-.---..T .A........ .......... ..........     119
acanthicum  -.--A.-.TT T...---..T .A........ .--------- -----.....     113

Consensus   GTTGTMWTKY AAGTKATGAY GGAYTWGCYG ATGAACTTTT RTTTTWMAMA     200
ultimum     .....AA.GC ....T....T ...C.A..T. .......... G....AA.--     184
aphanider.  .--------- ---------- -------.C. ...------. A....TC.A.     137
acanthicum  ..-..CT.TT ---.G...-C ...T.T..T. ...-----.. A....AA.C.     153

Consensus   CCYATTTACY TAAATACTGA WCTATACTSY RRRDACGAAA GTYYWTGSTT     250
ultimum     ..C--....C .......... T-......GT GGGG...... ..CCT..C..     231
aphanider.  ..C.....C .-........ T.......CC AAAA...... ..TTA..G..     186
acanthicum  ..T.-....T .-........ A.......CC GAAT...... ..TTT..G..     201

Consensus   TTAMYMDWTA ACAACTTTCA GCAGTGGATG TCTAGGCTCG CGC           293
ultimum     ...CTAGA.. .......... .......... .......... ...         274
aphanider.  ...ATCTA.. .......... .......... .......... ...         229
acanthicum  ...ACAAT.. .......... .......... .......... ...         244
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Fig. 2. Sequence alignment of the internal transcribed spacer I region of *Pythium ultimum* var. *ultimum* (CBS 398.51), *P. aphanidermatum* (CBS 118.80), and *P. acanthicum* (CBS 377.34). Gene-coding regions for the 18S and 5.8S genes are shaded. Consensus nucleotides are identified as dots, and sequence gaps are shown as dashes. The sequences used to synthesize the oligonucleotides in this study are underlined.

of that group but not to any of the other fungal genera tested (Fig. 3). It also hybridized to all *Phytophthora* and *Pythium* species tested (Figs. 4 and 5).

Several species were used to verify the genus specificity of the *Phytophthora* oligonucleotide. The *Phytophthora* species tested hybridized to the oligonucleotide, except *P. nicotianae* and *P. citricola* (Fig. 4). The intensity of the hybridization signal was lower with *P. cactorum* and *P. infestans*. Several isolates of *P. cinnamomi* were tested (Table 2), and all hybridized to the oligonucleotide designed for this species (Fig. 4).

Many isolates of the two varieties of *P. ultimum* were tested (Table 1), all of which hybridized to the oligonucleotide for *P. ultimum*, as shown in Figure 5. Isolates of *Pythium* group G also hybridized. None of the other species tested hybridized to this oligonucleotide (Fig. 5). All test isolates of *P. acanthicum* hybridized to the two oligonucleotides (92 and 132) designed for *P. acanthicum*. Oligonucleotide 132 also hybridized with *P. oligandrum* and *P. acanthophoron*, whereas the oligonucleotide 92 hybridized faintly to *P. acanthophoron*. All test isolates of *P. aphanidermatum* hybridized to the oligonucleotide designed for this species (Fig. 5). However, strong cross-hybridization was observed with *P. deliense* and *P. hypogynum*, and weaker reactions were observed with *P. aristosporum* and *P. arrhenomanes* (Fig. 5). Hybridization and washes done at 60°C showed that the *P. deliense* cross-reaction was still as strong as the reaction with *P. aphanidermatum*, while the others no longer cross-hybridized (Fig. 6). At this higher temperature, the universal oligonucleotide also did not hybridize.

The hybridization results were highly reproducible. Probes made from different PCR amplifications of the same isolates gave the same results with erased or newly blotted membranes. For selected isolates, the initial hybridization solution was reused with different membranes five consecutive times during a 4-month period. Results were highly consistent, but for each subsequent hybridization, film exposure had to be increased by a few minutes to achieve equivalent intensities.

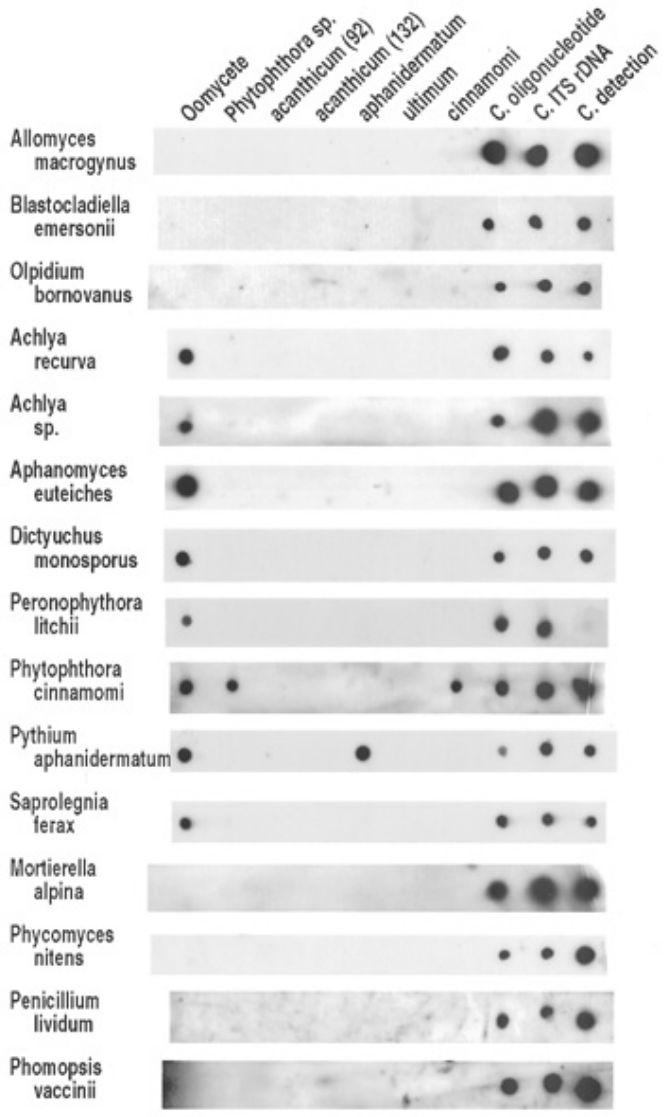

Fig. 3. Reverse dot blot hybridizations with immobilized specific oligonucleotides (listed at the top and in Table 2) to demonstrate the specificity of the oomycete and genus *Phytophthora* oligonucleotides (first two dots on the left of each membrane). The species listed on the left were used to make the probes for each hybridization (Table 1 has isolate numbers). Hybridizations and washes were performed at 55°C. The three dots on the right are controls (C.): oligonucleotide = universal ITS2 and ITS4 (Table 2); ITS rDNA = amplified and mixed ITS I from a wide range of genera; and detection = ITS I amplified and labeled with alkaline stable DIG-dUTP (digoxigenin-11-dUTP) (the membrane used for *Pyrenophythora litchii* was blotted at that location with an alkaline labile DIG-dUTP by mistake).

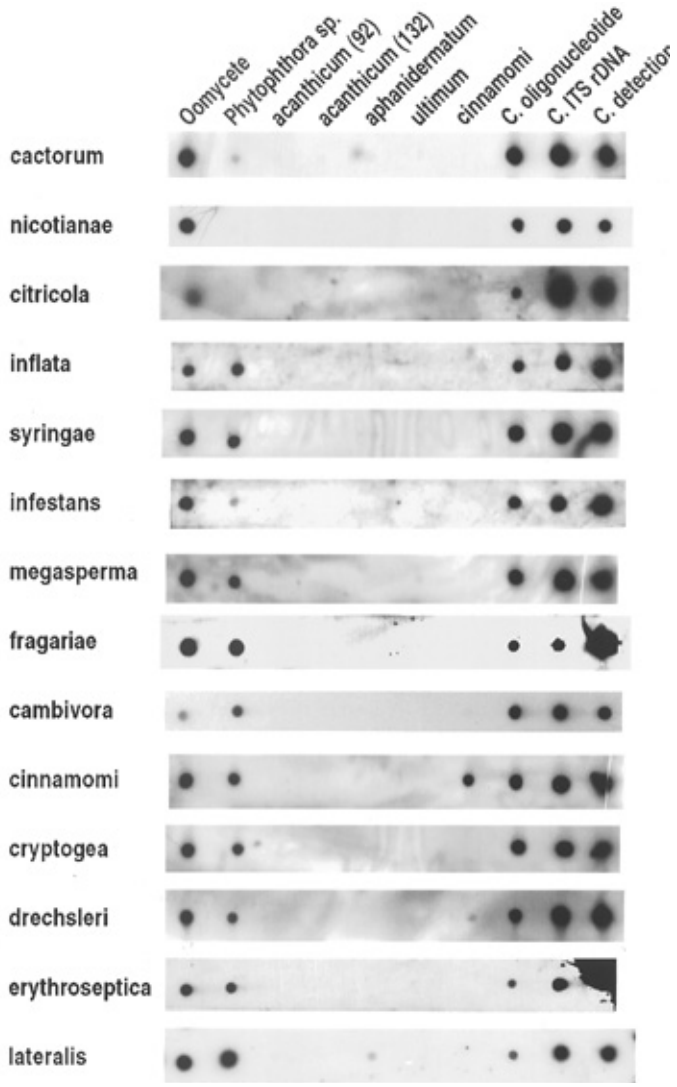

Fig. 4. Reverse dot blot hybridizations with immobilized specific oligonucleotides (listed at the top and in Table 2) to demonstrate the specificity of the genus *Phytophthora* and the *P. cinnamomi* oligonucleotides (second and seventh dots of each membrane). The *Phytophthora* species listed on the left were used to make the probes for each hybridization (Table 1 has isolate numbers). Hybridizations and washes were performed at 55°C. The three dots on the right are controls (C.): oligonucleotide = universal ITS2 and ITS4 (Table 2); ITS rDNA = amplified and mixed ITS I from a wide range of genera; and detection = ITS I amplified and labeled with alkaline stable digoxigenin-11-dUTP.

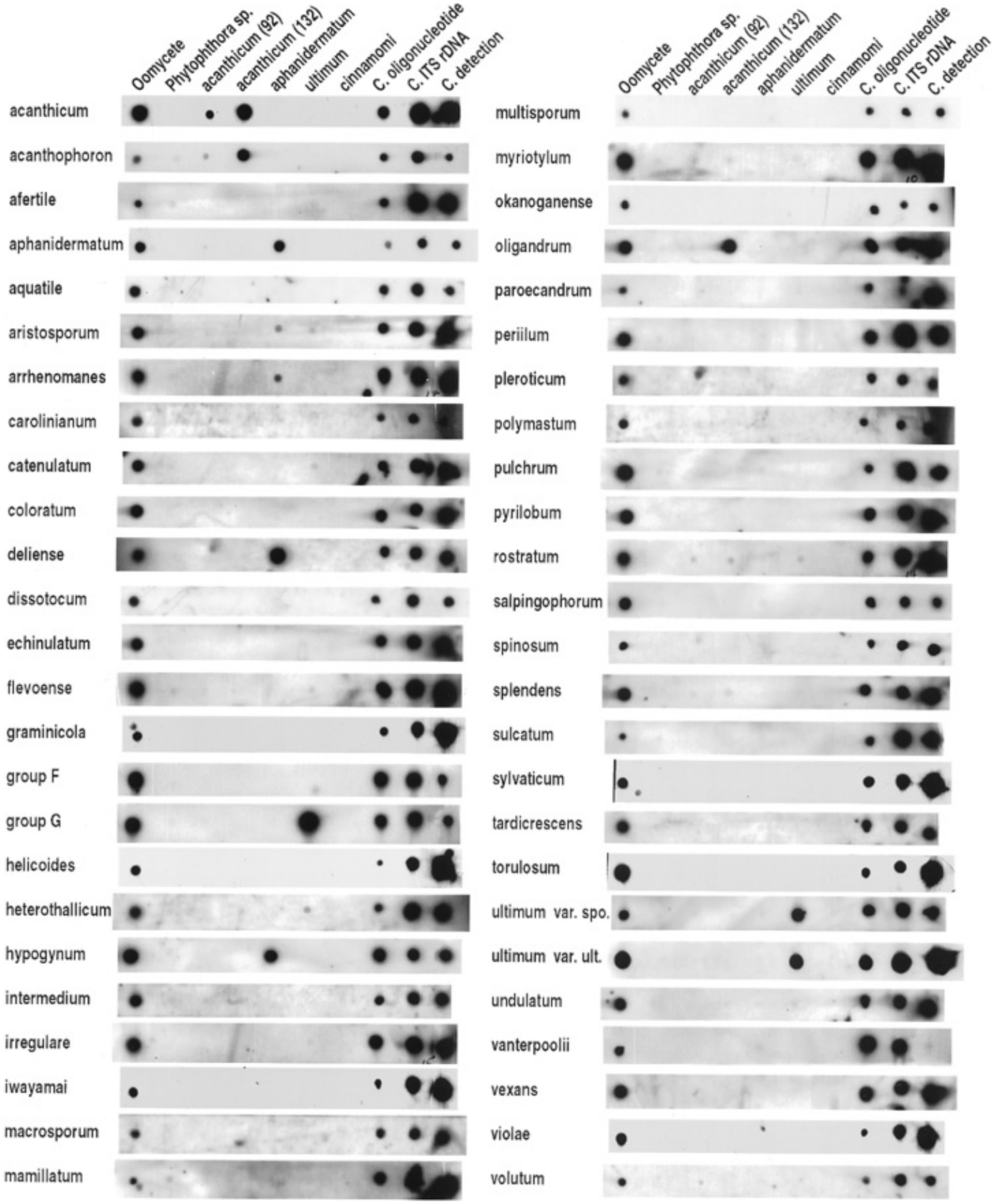

Fig. 5. Reverse dot blot hybridizations with immobilized specific oligonucleotides (listed at the top and in Table 2) to demonstrate the specificity of the *Pythium acanthicum*, *P. aphanidermatum*, and *P. ultimum* oligonucleotides. The *Pythium* species listed were used to make the probes for each hybridization (Table 1 has isolate numbers). Hybridizations and washes were performed at 55°C. The three dots on the right are controls (C.): oligonucleotide = universal ITS2 and ITS4 (Table 2); ITS rDNA = amplified and mixed ITS I from a wide range of genera; and detection = ITS I amplified and labeled with alkaline stable DIG-dUTP (digoxigenin-11-dUTP) (the membrane used for *P. vanterpoolii* was blotted at that location with an alkaline labile DIG-dUTP by mistake).

DISCUSSION

Two variations of the reverse dot blot technology were tested to identify oomycetes. The reverse dot blot with the immobilized entire ITS I is quite simple to develop and shows potential for identifying some species when pure cultures are used. This approach is a logical continuation of previous work by Lévesque et al. (30), where it was shown that the entire ITS I of *P. ultimum* could be used as a species-specific probe in standard dot blot assay. Positive results for *P. ultimum* (Fig. 2C) in the reverse dot blot assay with entire ITS I support these earlier findings. However, the reverse dot blot technique must be amenable not only to the identification of pure cultures, but ultimately to the direct processing of environmental samples from sources such as spore traps, soil, roots, and water. Patterns observed in reverse dot blots designed with the entire ITS I may prove useful to fully or partially identify a pure culture but will not work with field samples that might contain a mixture of species. For this reason, the reverse dot blot approach with the entire ITS I was not pursued further. Klassen et al. (23) showed that the 5S ribosomal RNA gene spacer could be used as a species-specific hybridization probe. This spacer might lend itself better to conversion to the reverse dot blot technique with entire species-specific PCR products immobilized on membranes.

Reverse dot blot with immobilized oligonucleotides gave much less ambiguous results than reverse dot blot with immobilized PCR products. All the species targeted in this study were identified by the reverse dot blot assay. Work with the cystic fibrosis gene has shown that a single base mismatch is enough to design a mutation-specific oligonucleotide for reverse dot blot (22). This is potentially a problem if one wants to be certain that a given oligonucleotide covers the range of variation in a species. To minimize this possibility, the species-specific oligonucleotides in this study were tested against isolates from different continents.

The oomycete oligonucleotide hybridized to all oomycete species tested but not to other zoosporic or coenocytic fungi. However, before it can be concluded that this is an oomycete-specific probe, its hybridization capability has to be confirmed in tests with representatives of groups that were not included in this study, such as the Leptomitales or downy mildews. The phylum Oomycota is now classified in the kingdom Stramenopila (2). Available ITS sequence data of stramenopiles (21,39) that do not belong to Oomycetes show as much dissimilarity with the oomycete-specific oligonucleotide sequence as does the genus *Penicillium* (32), which did not hybridize at all with the oomycete oligonucleotide. On the basis of the sequence differences, it is unlikely that the oomycete oligonucleotide would have hybridized to other stramenopiles.

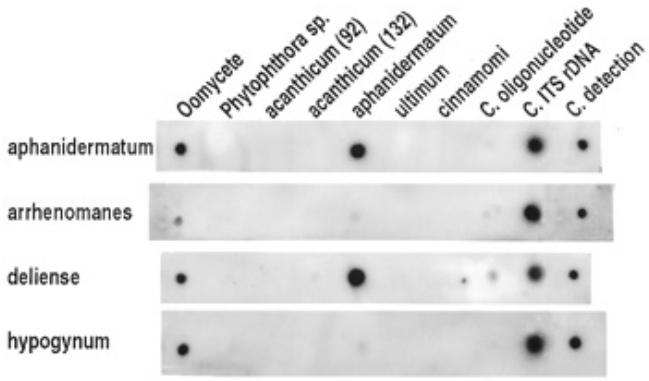

Fig. 6. Reverse dot blot hybridizations with immobilized specific oligonucleotides (listed at the top and in Table 2) to demonstrate the specificity of the *Pythium aphanidermatum* oligonucleotide. The *Pythium* species listed on the left were used to make the probes for each hybridization (Table 1 has isolate numbers). Hybridizations and washes were performed at 60°C. The three dots on the right are controls (C.): oligonucleotide = universal ITS2 and ITS4 (Table 2); ITS rDNA= amplified and mixed ITS I from a wide range of genera; and detection = ITS I amplified and labeled with alkaline stable digoxigenin-11-dUTP.

The oligonucleotide developed for the genus *Phytophthora* hybridized with most *Phytophthora* species tested. It is noteworthy that this oligonucleotide reacted strongly only with *Phytophthora* species with nonpapillate sporangia, except *P. inflata*, which has semipapillate sporangia. The oligonucleotide for the genus *Phytophthora* developed by Lee et al. (27) was hybridized by reverse dot blot, but its melting temperature was too low for the final conditions adopted (data not shown). Sequence extension of this oligonucleotide was not possible because of sequence heterology among species of *Phytophthora*. This oligonucleotide was located at the 3′ end of the ITS I spacer, whereas the one designed for the present study was located at the 5′ end. *P. cactorum* and *P. infestans* consistently gave the same faint hybridization reaction with the *Phytophthora* genus oligonucleotide developed for this study. The sequences of *P. infestans* published by Cooke et al. (12) after the present study was completed showed that our genus *Phytophthora* oligonucleotide had one mismatch six bases from the 5′ end. The *P. cactorum* sequence from Cooke et al. (12) showed the same mismatch as well as another mismatch at the final base of the 5′ end. The genus *Phytophthora* oligonucleotide from Lee et al. (27) had two consecutive mismatches at the far 3′ end for *P. cactorum* but still hybridized to this species in standard dot blot assay. Likewise, the additional mismatch at the far 5′ end for *P. cactorum* did not appear to decrease the hybridization signal compared with that observed with *P. infestans*, which had only one mismatch closer to the middle (Fig. 4). These observations would support the conclusion by Kawasaki et al. (22) that mismatches in the middle of an oligonucleotide are more destabilizing than those at the 5′ or 3′ end.

All isolates of *P. ultimum* hybridized to the oligonucleotide developed for this species, including isolates from *P. ultimum* var. *sporangiiferum* and *Pythium* "group G." This was consistent with standard dot blot results obtained previously (30). The results suggest that the isolates identified as *Pythium* group G actually represent sexually sterile *P. ultimum* isolates (37). Using three different molecular techniques, Francis et al. (15) concluded that the two varieties of *P. ultimum* were not genetically distinct. Using 5S ribosomal RNA gene spacer probes, Klassen et al. (23) found that there were two molecular groups within *P. ultimum* but that these groups did not correlate well with the variety classification (i.e., half the *P. ultimum* var. *sporangiiferum* isolates were similar to *P. ultimum* var. *ultimum* for molecular markers). Cross-reactions between the varieties were also observed on overexposed blots (23). Our partial reverse dot blot results with amplified PCR fragments also support the fact that there are at least two molecular groups for *P. ultimum* (Fig. 1C).

For identification of *P. acanthicum*, oligonucleotide 132 reacted strongly with *P. acanthicum*, *P. acanthophoron*, and *P. oligandrum*, while oligonucleotide 92 reacted only to *P. acanthicum* and *P. acanthophoron*. This combination of oligonucleotides can be used to identify either *P. oligandrum* or *P. acanthicum*. These results are consistent with the fact that *P. oligandrum* shares significant characteristics with *P. acanthicum*: both have ornamented oogonia and contiguous sporangia (40). The hybridization reactions of *P. acanthophoron* were more like those of *P. acanthicum* than *P. oligandrum*, although hybridization with oligonucleotide 92 was faint. Morphologically, *P. acanthophoron* is very similar to *P. acanthicum*, except that it lacks the ability to produce sporangia in culture (40).

The oligonucleotide developed for *P. aphanidermatum* cross-hybridized with some other *Pythium* species at 55°C, but when the assay temperature was increased to 60°C, cross-hybridization with only *P. deliense* was observed. In the taxonomic treatise of *Pythium* species by Van der Plaats-Niterink (40), these two spe-

cies are separated mainly by the curvature of the oogonial stalk in *P. deliense*. On the basis of present molecular and morphological evidence, it appears that the taxonomic status of these two species needs further clarification. The best results for identification of *P. aphanidermatum* were obtained at a higher temperature than that used for the other oligonucleotides, which poses a technical problem. However, we demonstrated that it is possible to obtain the same results as those shown in Figures 5 and 6 from a single hybridization done at 55°C (data not shown). After initial washes at 55°C followed by detection, more stringent washes and another detection can be performed on the same membrane.

One of the main advantages of the reverse dot blot technique is the potential for the integration of results from several other studies into a single assay. This is exemplified by the rapid expansion of the cystic fibrosis mutation screening assay (42). With the species-specific oligonucleotide for *Phytophthora cinnamomi* from Lee et al. (27), we have also demonstrated that the results from standard dot blot with oligonucleotides can be directly applicable to reverse dot blot. However, as shown with the example of the oligonucleotide for all *Phytophthora* species from Lee et al. (27), it will not always be possible to use oligonucleotide data directly from the work of others. As its name indicates, the reverse dot blot can use immobilized standard dot blot probes. Normally, in a standard dot blot assay, each of these oligonucleotide probes would be used at its own optimized hybridization condition. However, in a reverse dot blot assay, the oligonucleotide probes must all be used under the same conditions. The necessity for standardization of the melting temperatures in the design of all the oligonucleotides continues to be the principal technical difficulty and the main reason that species-specific oligonucleotides, used successfully in standard dot blot, have to be modified before use with this new technology. Equations for estimating melting temperature do not always predict accurately the optimal hybridization condition (27). Furthermore, the optimal standard dot blot hybridization condition for a given oligonucleotide may not match the optimal condition required for reverse dot blot (data not shown). Initial screening of oligonucleotides by standard dot blot was abandoned for this reason. The hybridization kinetics of the standard dot blot are probably quite different from those of a reverse dot blot. In the former, a short, free-floating molecule must bind and stay attached to a long, immobilized one; and in the latter, it is the short molecule that is immobilized to bind to the free, long ones. With the current increased usage of reverse dot blot in the medical field, it is hoped that more will become known about the theoretical melting point to facilitate the design of oligonucleotides for this technology.

We have demonstrated that the reverse dot blot technology can be used for identification of some oomycete species. There are numerous possibilities for expanding and refining this technology further. We have used only one pair of PCR primers to generate the labeled ITS region. Reverse dot blot has been used by labeling entire bacterial genomes (24) or by using several pairs of primers in a multiplex PCR reaction (22). As the GenBank database increases (5) and as insightful interpretations of the data allow updates of the classification, integration of more organisms into identification systems should become possible. For example, if sets of species-specific oligonucleotides from mitochondrial and nuclear DNA exist for a given group of fungal species, it would be possible to have a reverse dot blot assay that would simultaneously identify and validate a given isolate with these two very different sets of oligonucleotides. Primers targeting genes for avirulence or fungicide tolerance with nested specific oligonucleotides could also be incorporated in a reverse dot blot assay to go beyond species identification. Existing technologies can already overcome the physical limitations related to the number of specific oligonucleotides that can be blotted on a single membrane. More than one million different oligonucleotides have been synthesized directly on a silicon surface (31). The "lab in a microchip" design, being pursued by several companies, will drastically speed up sample processing for hybridization to oligonucleotide arrays and is likely to reduce cost as well (34). Since the first paper in 1989 describing hybridization to immobilized arrays of oligonucleotides (36), the number of papers in the medical field has almost doubled every year, reaching 30 in 1995. The majority of these papers describe detection of genetic disorders, but there is an increasing number of papers that focus on detection and identification of pathogenic bacteria or viruses (1,20). With more complex arrays, the reverse dot blot technology could be useful for routine identification of fungi, diagnosis of plant diseases, and detection of fungi in soil or spore traps. The opportunities for integrated disease management arising from the applications of new detection technologies were recently reviewed (28).

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