Development of a Species-Specific Probe for *Pythium ultimum* Using Amplified Ribosomal DNA

C. André Lévesque, Thierry C. Vrain, and Solke H. De Boer

Agriculture Canada Research Station, Vancouver, BC, V6T 1X2.
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ABSTRACT

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The internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) was amplified by the polymerase chain reaction (PCR) with universal primers and used to differentiate species of *Pythium* that are difficult to identify by morphological criteria. The restriction sites on the ITS region of an isolate of *Pythium ultimum* were mapped. Digoxigenin-labeled probes (100–200 bp) representing different sequences along the entire ITS region were prepared from gel-purified restriction fragments of rDNA amplified by PCR. The restriction fragment probes from the ITS I spacer between the small ribosomal DNA (SrDNA) subunit and the 5.8S gene had a high degree of species specificity to *P. ultimum* when tested by dot blot hybridization against 24 other *Pythium* species. Similar results were obtained when the entire ITS I of *P. ultimum* was used as a probe. The quantities of total DNA on dot blots were standardized at 5 or 50 ng per dot. No difference in hybridization could be detected among the 13 isolates of *P. ultimum* (var. *ultimum* and var. *sporangiferum* from eight countries) and two isolates of *Pythium* group G recently classified as *P. ultimum*.

Additional keywords: chemiluminescence, diagnosis, DIG-dUTP, NIH Image, T4 DNA polymerase.

The taxonomy of Oomycetes is based almost exclusively on morphological characteristics (4,22) that are highly variable and make identification of species within this group difficult and often questionable (10). The plasticity among isolates of a single *Pythium* species is such that large variations can be observed in the parameters measured for identification (2). Confusion in the taxonomy and/or difficulties in routine identification of these species result in limitations in certification of planting stock, breeding programs, and ecological or biological control studies.

Techniques in molecular biology are being used to better understand the relationship and the evolution of species (5,15). Separation of proteins by electrophoresis has been used to differentiate *Pythium* species (7). The antibody-based commercial kits presently available can identify only to the genus level (1). Restriction fragment length polymorphism (RFLP) of ribosomal DNA (rDNA) has been used to differentiate certain *Pythium* species (8). Amplification by the polymerase chain reaction (PCR) followed by digestion with restriction enzymes has been used to study variation in the internal transcribed spacers (ITS) of the rDNA cistron and relationships among three *Pythium* species (6). Oligonucleotide probes for *Phytophthora* species were synthesized by comparing the sequences of the ITS I for different species in this genus (16,17). Species-specific genomic DNA probes selected from random clones also have been made for *Phytophthora citrophthora* and *Phytophthora parasitica* (11–13). There is a need for a generalized approach that can be used for rapid production of species-specific probes and for a technique that does not require sequencing or production of a genomic DNA library.

The objective of this study was to find a DNA fragment in the ITS region between the small rDNA (SrDNA) and the large rDNA (LrDNA) subunits that could be used as a species-specific probe for *Pythium ultimum* Trow in routine identification by nonisotopic dot blot hybridization.

MATERIALS AND METHODS

Fungal collection. A collection of 46 isolates of *Pythium* species that included 24 *Pythium* species was used in this study (Table 1). The 21 isolates from the Centraalbureau voor Schimmelculture (Baarn, the Netherlands) are neotype cultures used to describe the species in a *Pythium* monograph (22). Cultures of *Pythium* species were maintained on slants under mineral oil and in water (9). An isolate of *Sclerotium rolfsii* Sacc. (from Z. K. Punja, Simon Fraser University, Burnaby, BC, Canada) also was used.

Fungal growth and DNA extraction. All *Pythium* isolates were grown on water agar with streptomycin and penicillin (50 and 100 units ml^{-1}, respectively) before transfer to a broth medium. V8 broth medium without cholesterol (3) was filtered successively through a 15-cm Whatman No. 1 filter paper (Maidstone, England) and a 7.5-cm Whatman grade A glass-fiber filter before autoclaving. Agar plugs (8 mm diameter) taken from the margins of colonies on water agar were used as inoculum. DNA was extracted from 2-day-old cultures as previously described (18). After a first ethanol precipitation, pellets were resuspended in 400 μl of TE buffer (10 mM Tris, pH 8.0, 1 mM Na_2EDTA) with RNase (10 $\mu g\ ml^{-1}$), incubated at 37 C for 30 min, and reprecipitated. Pellets were resuspended in TE and diluted until total DNA concentration reached approximately 400 ng μl^{-1}. Concentrations of total DNA were estimated by electrophoresis with calf-thymus DNA standards followed by densitometry of a scanned negative of the gel by NIH Image software (20).

Restriction map. The ITS region was amplified with PCR, using the primers ITS1 and ITS4 (25) with approximately 50 ng of total DNA from *P. ultimum* (IMI 334959) as template. Ribosomal DNA from IMI 334959 purified by CsCl centrifugation was used as a control in all DNA amplifications. The PCR reagents were obtained from a kit (Perkin-Elmer Cetus, Branchburg, NJ). An Ericomp (San Diego, CA) thermocycler was used for 40 cycles of 1 min at 94 C, 1 min at 55 C, and 2 min at 72 C. Approximately 0.5–1.0 μg of DNA from the PCR product was digested for 2 h at 37 C with 2.5–5.0 units of one of the following enzymes: *Alu*I, *Ava*I, *Ava*II, *Cfo*I, *Cla*I, *Dde*I, *Eco*RI, *Hae*III, *Hin*dIII, *Hin*fI, *Hpa*I, *Kpn*I, *Mbo*I, *Pst*I, *Pvu*I, *Rsa*I, *Sma*I, *Taq*I, *Xba*I, *Xho*I

(obtained from various suppliers). The DNA was size separated in 2% agarose gel at 3.3 V cm^{-1} (21). The 70- to 1,000-bp fragments from a 1-kb DNA ladder (Gibco BRL, Gaithersburg, MD) were used as size markers. The gels were photographed over a 300-nm UV light source to record DNA restriction band patterns. Once an enzyme was able to cut the DNA, the restriction fragments from three different amplified PCR products, ITS I, ITS II, and the two ITS combined (25), were compared to deduce the restriction sites for each enzyme. Partial digestion with an incubation time of 20 min or digestions with pairs of restriction enzymes were done when necessary to determine the location of some restriction sites. All digests were done at least twice.

Probe purification and labeling. Once the restriction map was defined, fragments A–F (Fig. 1) were gel-purified with 2% low-melting agarose (25 cm of gel, 3.3 V cm^{-1}) and a hot phenol extraction procedure (19). The following restriction fragments were produced after amplification by PCR and digestion of 20–30 μg of DNA: fragment A was obtained from a digest with *Dde*I; fragment B was obtained from a digest with *Cfo*I and *Taq*I; fragment C was obtained from a digest with *Hin*fI; and fragments D–F were obtained from a digest with *Mbo*I, *Rsa*I, and *Ava*II. When the complete ITS I was used as a probe, it was gel-purified with the Magic PCR Preps DNA purification system (Promega, Madison, WI) after amplification. Approximately 100 ng was end-labeled with T4 DNA polymerase and digoxigenin (DIG-dUTP), using 200 μM dATP, dCTP, and dGTP; 16.7 μM dTTP; and 8.3 μM DIG-dUTP. The duration of the exonuclease part of the reaction was determined for each fragment by dividing

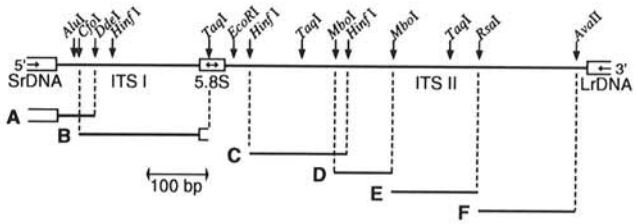

Fig. 1. Restriction map of the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) region of *Pythium ultimum* (IMI 334959) and location of the fragments used as probes in the dot blot hybridization study. The locations and sizes of the small subunit (SrDNA), large subunit (LrDNA), and 5.8S genes are based on sequences of *Phytophthora megakarya* (16).

TABLE 1. Isolates of *Pythium* species used in this study

Species	Source[a]	Number	Host	Location
P. ultimum var. ultimum	IMI	334959	*Solanum tuberosum*	Peru
P. u. ultimum	CBS	291.31	*Dioscorea batatas*	France
P. u. ultimum	CBS	296.37	*Pisum sativum*	U.K.
P. u. ultimum	CBS	488.86	*Malus sylvestris*	Poland
P. u. ultimum	CBS	398.51	*Lepidium sativum*	Netherlands
P. u. ultimum	CAL	AA2	*Phaseolus vulgaris* in loam soil	Canada, BC
P. u. ultimum	CAL	AA6	*Triticum aestivum* in loam soil	Canada, BC
P. u. ultimum	CAL	AB45	*Triticum aestivum* in muck soil	Canada, BC
P. u. ultimum	RD	R8	*Phaseolus vulgaris* in mineral soil	Canada, BC
P. u. ultimum	CAL	AB15	*Triticum aestivum* in muck soil	Canada, BC
P. u. sporangiiferum	CBS	219.65	*Chenopodium album*	U.S.
P. u. sporangiiferum	CBS	171.68	*Medicago sativa*	U.K.
P. u. sporangiiferum	CBS	111.65	*Medicago sativa*	Lebanon
Pythium group G	RD	R37	*Phaseolus vulgaris* in loam soil	Canada, BC
Pythium group G	RD	R51	*Phaseolus vulgaris* in muck soil	Canada, BC
P. acanthicum	DJSB	B 500	Soil	Canada, ON
P. acanthicum	CBS	377.34	*Solanum tuberosum*	Sweden
P. aphanidermatum	DJSB	B 444	*Cucumis sativus*	Canada, BC
P. aphanidermatum	CBS	118.80	Unknown	France
P. aphanidermatum	DJSB	B 206	*Pinus resinosa*	Canada, ON
P. arrhenomanes	DJSB	B 671	*Avena fatua*	Canada, MB
P. coloratum	DJSB	B 401	*Medicago sativa*	Canada, ON
P. dissotocum	CBS	166.68	*Triticum aestivum*	U.S.
P. graminicola	BCMAFF	92-084-8	Turfgrass	Canada, BC
P. heterothallicum (male)	CBS	450.67	*Sambucus* species	Canada
P. heterothallicum (female)	CBS	451.67	*Sambucus* species	Canada
P. hypogynum	DJSB	B 393	*Chenopodium album*	Canada, PQ
P. intermedium	EH	5159	Water baiting	SE Alaska
P. irregulare	CBS	250.28	*Phaseolus vulgaris*	Netherlands
P. irregulare	RD	R12	*Phaseolus vulgaris* in loam soil	Canada, BC
P. irregulare	CAL	AB49	*Triticum aestivum* in loam soil	Canada, BC
P. iwayamai	CBS	156.64	Soil under *Pinus* species	Australia
P. multisporum	CBS	470.50	Soil	U.S.
P. okanoganense	CBS	315.81	*Triticum aestivum*	U.S.
P. oligandrum	CBS	382.34	*Viola* species	U.K.
P. paroecandrum	CBS	157.64	Loam soil	Australia
P. pyrilobum	CBS	158.64	*Pinus radiata*	Australia
P. rostrarum	CBS	172.68	*Medicago sativa*	U.K.
P. splendens	DJSB	B 435	*Vaccinium* species	Canada, NS
P. sulcatum	DJSB	B 195	*Daucus carota*	Canada, BC
P. sylvaticum (male)	CAL	AA4	*Triticum aestivum* in muck soil	Canada, BC
P. sylvaticum (female)	CAL	AB4	*Phaseolus vulgaris* in muck soil	Canada, BC
P. sylvaticum (male)	CAL	AB9	*Triticum aestivum* in loam soil	Canada, BC
P. torulosum	BCMAFF BR	727-2	Turfgrass	Canada, BC
P. undulatum	EH	5183	Water baiting	SE Alaska
P. violae	CBS	178.86	*Daucus carota*	Netherlands

[a]IMI = International Mycological Institute, Kew, England; CBS = Centraalbureau voor Schimmelculture, Baarn, the Netherlands; CAL = collection of C. A. Lévesque, Agriculture Canada Research Station, BC; RD = collection of R. Descalzo, Simon Fraser University, Burnaby, BC, Canada; EH = collection of E. Hansen, Oregon State University, Corvallis; BCMAFF = collection of B.C. Ministry of Agriculture, Fisheries and Food, Cloverdale, Canada; DJSB = collection of D. J. S. Barr, Biosystematics Research Center, Ottawa, Canada.

the estimated number of base pairs by 60. The protocol of the manufacturer of T4 DNA polymerase (Gibco BRL, Gaithersburg, MD) was followed until the addition of the stop buffer, after which the protocol of the manufacturer of DIG-dUTP (Boehringer Manheim, Germany) was followed for removal of unincorporated nucleotides. The entire ribosomal cistron from *Caenorhabditis elegans* (from D. L. Baillie, Simon Fraser University, Burnaby, BC, Canada) was used as a universal probe, and 100 ng was labeled, using the random primer kit from Boehringer Manheim. One-half of the labeled-DNA aliquot was used as a probe.

Dot blot hybridization and digoxigenin detection. Total DNA aliquots were diluted to adjust concentrations and were mixed to an equal amount of 1.0 M NaOH for denaturation. For hybridization with restriction fragments, 1-μl droplets (50 ng μl^{-1} of DNA) were placed on a Genebind (Pharmacia, Uppsala, Sweden) nylon membrane in a 1-cm grid pattern. For final hybridization with a species-specific probe, 1-μl droplets (5 ng μl^{-1} of DNA) were placed in a 8-mm grid pattern on a nylon membrane (Boehringer). Hybridizations were done at 68 C in 10-ml volumes; the procedure of Boehringer Manheim was used with high-stringency washes at 68 C in 0.1× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0). Hybridization and washes with the entire ITS I also were done at 80 C. Digoxigenin was detected by chemiluminescence, using 3-(2′spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane (Lumigen-PPD, Boehringer) with 1–3 h of film exposure (Kodak X-Omatic K) for restriction fragment probes and 15 min for the *C. elegans*

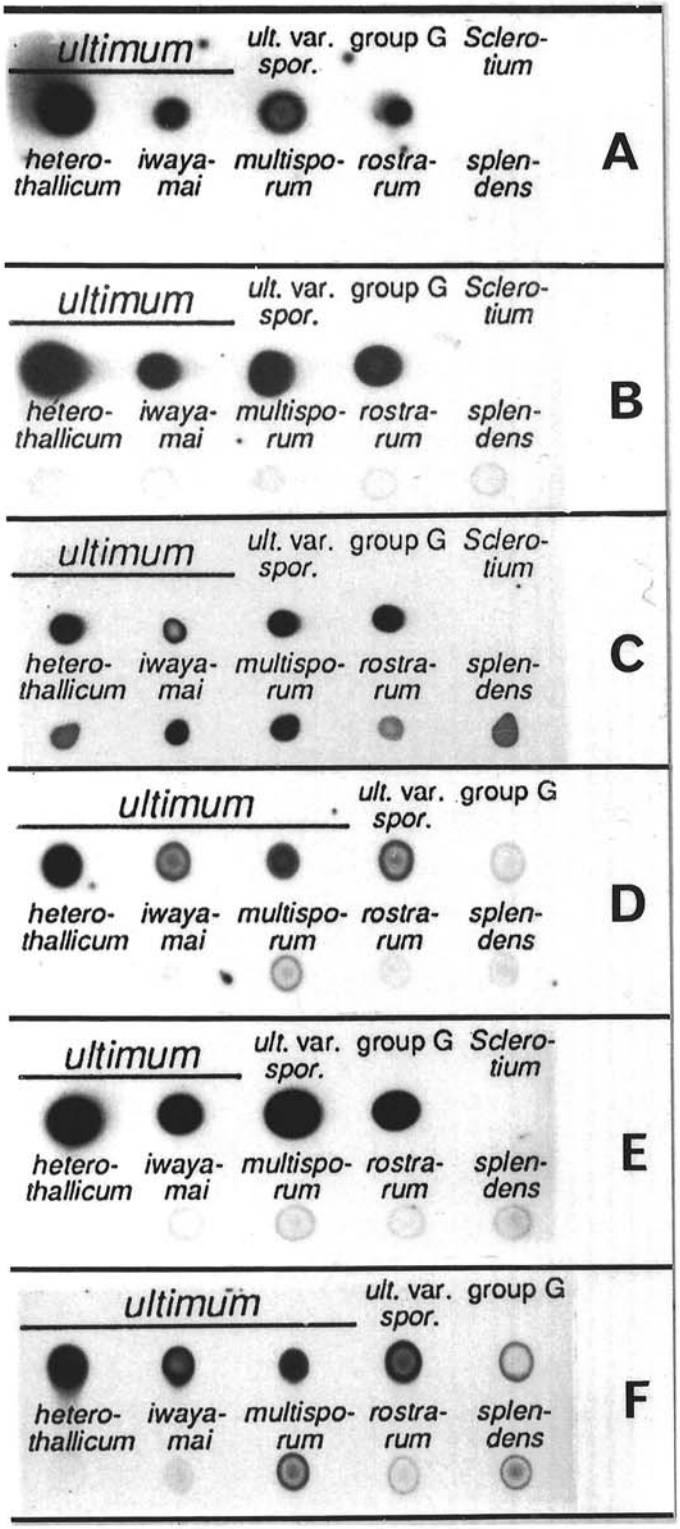

Fig. 2A–F. Dot blot hybridizations (50 ng of total DNA per dot) for six probes made from different restriction fragments of the internal transcribed spacer region of an isolate of *Pythium ultimum* from Peru (top left dot). The one or two *P. ultimum* isolates on each blot were from British Columbia, and the other dots represent different *Pythium* species, with the exception of a *Sclerotium rolfsii* isolate in **A, B, C,** and **E.** The letter for each hybridization also refers to the different restriction fragments represented in Figure 1.

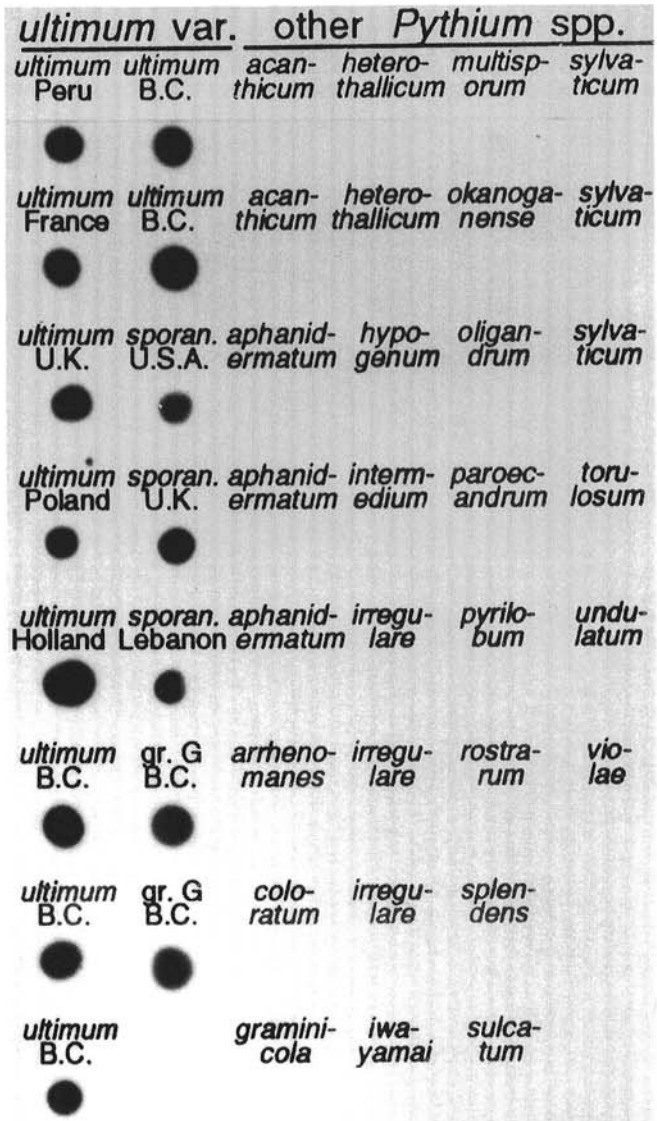

Fig. 3. Dot blot hybridization for the ITS I probe made by polymerase chain reaction with the neotype isolate of *Pythium ultimum* var. *ultimum* from Holland as template and T4 DNA polymerase for digoxigenin labeling. There was 5 ng of total DNA per dot. The film was exposed for 15 min to the membrane after the chemiluminescence reaction.

probe or the probe made of the entire ITS I. All hybridizations were done at least twice.

RESULTS

Restriction map. Ten restriction enzymes out of the 20 tested cut within the nuclear rDNA ITS region of *P. ultimum* generated by the PCR reaction (Fig 1). *Cla*I also could cut within the 5.8S primer sequence, at the same location as *Taq*I (25). The location and size of the genes is based on the distance between the primers and the end of the genes in *Phytophthora megakarya* (16).

Probes for *P. ultimum*. The fragments A–F (Fig. 1), derived from a Peruvian isolate of *P. ultimum*, were very different in their specificity for *P. ultimum* by dot blot hybridization (Fig. 2). Fragments A, B, and E were the most specific (Fig. 2A, B, and E). Fragment C was not species specific but did not cross-hybridize with *S. rolfsii* (Fig. 2C). All the restriction fragment probes hybridized to the *P. ultimum* isolates from British Columbia, to *P. u.* var. *sporangiferum* Drechs., and to *Pythium* group G. Fragments D and F hybridized to other *Pythium* species (Fig. 2D and F). Because fragments A and B showed specificity to *P. ultimum*, the entire PCR-amplified ITS I was labeled and used as a probe. The PCR-amplified ITS I from the neotype culture of *P. ultimum* (CBS 398.51) was species specific under the hybridization conditions described (Fig. 3). Increasing the hybridization and washing temperature to 80 C with the ITS I probe gave the same results as those shown in Figure 3 if the exposure time was extended to 8 h.

Control hybridization with universal probe. The ribosomal cistron of *C. elegans* used as a universal probe hybridized with a uniform intensity to all *Pythium* species isolates. The average gray value of the total intensity for each dot was 1,540, with a standard error of 53. The average gray value of the total intensity of the individual dots of *P. ultimum*, including group G and var. *sporangiferum* (Fig. 1), was 907, with a standard error of 71. Therefore, the amount of target ribosomal cistron in total DNA aliquots appeared to be uniform between and within species.

Reproducibility of the method. The hybridization results shown in Figure 3 were reproduced six times with probes made from different PCR-amplification reactions and newly blotted membranes, which included heat-denatured DNA blotted on Genebind membranes. Results from Figure 3 were the same if the ITS I from the Peruvian isolate was used instead of the neotype isolate from the Netherlands. The first hybridization solution that was made was reused with new membranes five consecutive times during a 4-mo period. The results were always the same, except that the film-exposure time had to be increased by a few minutes every time to give equivalent intensity. All other hybridizations were repeated at least once and gave the same results.

DISCUSSION

There are DNA sequences in the ITS region unique to *P. ultimum* that appear to be conserved within this species. The ITS I probe hybridized to all other *P. ultimum* isolates even with hybridization and washes done at 80 C, which further supports the fact that the ITS I is highly conserved in isolates of *P. ultimum* geographically far apart. The ITS region appears to contain sequences useful for taxonomy and identification of Oomycetes. Chen et al (8) differentiated five *Pythium* species by endonuclease digestion of a PCR-amplified SrDNA and ITS region. Chen (6) was able to differentiate heterozygous *Pythium* species by RFLP of ITS I and ITS II. Lee and Taylor (16) sequenced ITS I for three *Phytophthora* species, and Lee et al (17) subsequently synthesized species-specific oligonucleotides derived from nonhomologous sequences within ITS I.

Isolates belonging to *Pythium* group G do not produce the oogonia essential in classical taxonomy of Oomycetes. Two recent studies with RFLP of repeated sequences of either mitochondrial DNA (14) or total DNA (18) have shown that *Pythium* group G is a variant of *P. ultimum*. The *P. ultimum* ITS I probe used here hybridized as strongly to isolates of *Pythium* group G as to any other isolates of *P. ultimum*. This is a third type of DNA comparison that supports the fact that *Pythium* group G is a different form of *P. ultimum*.

ITS probes for *P. ultimum* made with universal primers located further from the 3′ end of the SrDNA and from the 5′ end of the LrDNA (23), i.e., with more conserved gene sequence than the probe described here, showed a high level of cross-hybridization (data not shown). It might be possible to generate quickly species-specific probes for many different species with universal primers located immediately at each end of a variable sequence. This appears to be the case for some species of *Pythium* if the ITS1 and ITS2 primers (25) are used to generate a probe (data not shown). These results are preliminary and based on a limited number of species and isolates.

The new approach shown here has two main advantages over the techniques used previously to produce a species-specific probe: 1) it does not involve the production of a library from which clones containing species-specific DNA fragments are screened, and 2) there is no need for sequencing to produce the species-specific probe. Mitochondrial rDNA probes have been produced by PCR, but they had to be used with Southern blots to produce RFLP that could differentiate varieties and species within the *Gaeumannomyces-Phialophora* complex (24).

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