Identification of the Tobacco Blue Mold Pathogen, *Peronospora tabacina,* by Polymerase Chain Reaction

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ABSTRACT

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Tobacco blue mold, caused by the oomycete pathogen *Peronospora tabacina*, is a highly destructive pathogen of tobacco (*Nicotiana tabacum*) seed beds, transplants, and production fields in the United States. The pathogen also causes systemic infection in transplants. We used polymerase chain reaction (PCR) with the primers ITS4 and ITS5, sequencing, and restriction digestion to differentiate *P. tabacina* from other important tobacco pathogens, including *Alternaria alternata*, *Cercospora nicotianae*, *Phytophthora glovera*, *P. parasitica*, *Pythium aphanidermatum*, *P. dissotocum*, *P. myriotylum*, *P. ultimum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii, Thielaviopsis basicola*, and related *Peronospora* spp. A specific PCR primer, called PTAB, was developed and used with ITS4 to amplify a 764-bp region of DNA that was diagnostic for *P. tabacina*. The PTAB/ITS4 primers did not amplify host DNA or the other tobacco pathogens and were specific for *P. tabacina* on tobacco. DNA was detected to levels of 0.0125 ng. The PTAB primer was useful for detection of the pathogen in fresh, air-dried, and cured tobacco leaves. This primer will be useful for disease diagnosis, epidemiology, and regulatory work to reduce disease spread among fields.

Additional keyword: oomycetes

Blue mold is a devastating downy mildew disease of tobacco (*Nicotiana tabacum*) caused by the Oomycete pathogen *Peronospora tabacina* Adam (17,21,24,35). *P. tabacina* is an obligate parasite and cannot be cultured in vitro (17,22,35). Sporangiospores of *P. tabacina* do not produce zoospores, and infection occurs via direct germination (6,35). The asexual sporangiospores of the pathogen can be dispersed thousands of kilometers and are the primary source of inoculum for epidemics (7–9,35). In the United States, sporangiospores may be spread yearly to commercial tobacco from wild tobacco in Texas or may originate in Caribbean countries and move northward and infect tobacco as it is planted in fields (1,8,9,15,18,19,24,34). Tobacco blue mold is also a problem in southwestern and southeastern Europe, the Middle East, and North Africa. Inoculum may overwinter in North Africa and is dispersed long distances to southern Europe.

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The Tobacco Blue Mold Warning System, located at North Carolina State University in Raleigh, provides continent-wide Internet forecasting support to tobacco growers by tracking the geographic presence and future spread of the tobacco blue mold pathogen (19). In some years, tobacco blue mold has been very severe in some areas of the United States but not in the southernmost regions, as the forecasting trajectories might have predicted (19,28). Epidemics have been traced to inoculum from greenhouse transplants. Tobacco transplants that are infected by *P. tabacina* and taken to the field and planted may remain stunted, produce lower yields, or simply die (4,15). Infected transplants also can introduce the pathogen to noninfested areas and these plants then can act as a reservoir for subsequent epidemics in the field (31). It is unclear whether the pathogen is capable of overwintering in infected debris, and the role of oospores in disease is not clearly understood. Populations of the pathogen are resistant to the commonly used fungicides such as metalaxyl and mefenoxam (29). Tobacco blue mold cannot be controlled by crop rotation and there is only one cultivar of Burley tobacco that is resistant to the pathogen.

A reliable and quick method of detection of the pathogen could be useful to reduce spread of the pathogen in infected plants or to identify field or aerial sources of inoculum for forecasting systems. Diagnosis can be difficult when sporulation is not observed (4,17). A number of other fungal pathogens cause leaf spot and stem or systemic infections on tobacco, thus complicating diagnosis. The pathogen already may have released sporangiospores into the atmosphere by the time accurate identifications are made. Proper disease management may be problematic once a plant has become infected because most fungicides are protectants and not curative.

A polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay of nuclear and mitochondrial genes has been used to differentiate the major species of *Phytophthora* and strains that cause disease on different hosts (14,20,30). The internal transcribed spacer (ITS) regions and the 5.8S rDNA gene have been sequenced and used in systematics at the species level for many fungal and oomycete species. However, the ITS regions have not been sequenced in *P. tabacina*. Only a single PCR-based method using random amplified polymorphic DNA (RAPDs) has been developed for detection of *P. tabacina*, and no methods have been deployed for use in the field or thoroughly tested against other tobacco pathogens (4,38). Intraspecific variation has been studied with allozyme analysis and repetitive DNA probes among a limited number of isolates of *P. tabacina* (23,32).

The objectives of this work were to sequence the ITS and 5.8S rDNA of *P. tabacina* and other important fungal pathogens of tobacco and to develop a specific PCR assay for the in vivo detection of the pathogen in infected tobacco leaves.

MATERIALS AND METHODS

Growth of tobacco seedlings. Tobacco seed (cvs. Burley 21 and Bergerac) were planted in small plastic trays and germinated in a mist room. The seed trays were transferred to a growth chamber at 22ºC and were watered with Hoagland's nutrient solution (13). The seedlings were planted in small Styrofoam cups and moved to a 22ºC greenhouse once the true leaves were formed. The tobacco plants were watered twice daily with the Hoagland's nutrient solution and exposed to long days by interrupting the regular 15-h night period at 18ºC with 3 h of extra light in the middle of the dark period. Plants at the three- to four-leaf stage were placed in sealed plastic boxes and watered with 250 ml of Hoagland's solution

Table 1. Isolate name, year of isolation, geographic location (county or state) of source, and name of collector of isolates of *Peronospora tabacina* used in this study

Isolate[a]	Year	Source[b]	Collector[c]
BPTP[d]	1945	Texas	Clayton
BPTS	1945	Texas (subculture of BPTP)	Clayton
98 Kentucky	1999	Kentucky	P. Shoemaker
98 Madison	1999	Madison County, NC	P. Shoemaker
98 Virginia	1999	Virginia	P. Shoemaker
98 Watauga	1999	Watauga County, NC	P. Shoemaker
Ashe (UMRS)	1998	Ashe County, NC	P. Shoemaker
Brunswick Co.	1999	Brunswick County, NC	J. Radford
CT 99 1	1999	Connecticut	J. LaMondia
GA 99 1	1999	Doerun, GA	P. Bertrand
GA 99 2	1999	Colquitt County, GA	P. Bertrand
GA 99 4	1999	Reno, GA	P. Bertrand
GA 99 5	1999	Berlin, GA	P. Bertrand
GA 99 6	1999	Tifton, GA	A. Csinos
Greene City 1	1998	Greene County, NC	A. Johnson
Haywood Co.	1999	Haywood County, NC	P. Shoemaker
JPT84	1984	Jones, NC	M. Moss
KPT 79	1985	Kentucky	W. Nesmith
KY 98	1999	Kentucky	W. Nesmith
KY Nesmith	1999	Kentucky	P. Shoemaker
Lenoir Co.	1999	Lenior County, NC	C. E. Main
MC Holloway	1999	Madison County, NC	S. Holloway
Mexico	1999	Mexico	R. Rufty
Millersville, PA	1999	Millersville, PA	J. Yocum
Mitchell	1998	Mitchell County, NC	P. Shoemaker
Mitchell 98	1998	Mitchell County, NC	P. Shoemaker
Moseley VA, 1A	1999	Moseley, VA	C. Johnson
Moseley VA, 1B	1999	Moseley, VA	C. Johnson
MRS 97	1999	Haywood County, NC	P. Shoemaker
NC-99-10	1999	Buncombe County, NC	P. Shoemaker
NC-99-3	1999	Pitt County, NC	P. Shoemaker
NC-99-4[d]	1999	Robeson County, NC	P. Shoemaker
NC-99-6[d]	1999	Brunswick County, NC	P. Shoemaker
NC-99-7	1999	Jones County, NC	P. Shoemaker
NC-99-9[d]	1999	Madison County, NC	P. Shoemaker
NPT 83	1984	Northampton County, NC	P. Shoemaker
Onslow	1999	Onslow County, NC	K. Benson
OPT 79	1979	Oxford	H. Spurr
OPT 83	1983	Oxford	H. Spurr
OPT 84	1984	Oxford	H. Spurr
Columbia, PA[d]	1998	Columbia, PA	W. Nesmith
Pennsylvania A	1999	Leola, PA	J. Yocum
Pennsylvania B	1999	Smoketown, PA	J. Yocum
PT 3589 A	1989	Buncombe County, NC	P. Shoemaker
PT 3589 F	1989	Madison County, NC	P. Shoemaker
PT 3589 K	1989	North Carolina	P. Shoemaker
PT 3589 L	1989	Yancey County, NC	P. Shoemaker
PT 3589 M	1989	Yancey County, NC	P. Shoemaker
PT 86 GH	1999	Yancey County, NC	P. Shoemaker
PT 87 W	1999	Weaverville County, NC	P. Shoemaker
PT 87 Y[d]	1998	Yancey County, NC	P. Shoemaker
PT 88 W	1999	Haywood County, NC	P. Shoemaker
PT 89 B	1989	Buncombe County, NC	P. Shoemaker
PT91A	1991	Allegheny, NC	G. Atwood
PT97H	1997	…	P. Shoemaker
PT W 87	1999	Weaverville County, NC	P. Shoemaker
PTMRS 97	1999	North Carolina	P. Shoemaker
PTNC 96	1998	Bladen County, NC	P. Shoemaker
Stokes	1993	Stokes, NC	Loudermilk
Surry Co.	1999	Surry County, NC	P. Shoemaker
VA 98	1998	Virginia	F. Bolick
Virginia	1998	Blackstone, VA	C. Johnson
Waynesville 1	1999	Haywood County, NC	P. Shoemaker
Waynesville 2	1999	Haywood County, NC	P. Shoemaker
Waynesville 3	1999	Haywood County, NC	P. Shoemaker
WC Bolick	1998	Watauga County, NC	P. Shoemaker

[a] Isolate name and year of collection.
[b] Source indicates county and state where samples were collected.
[c] Name of collector.
[d] DNA from these isolates were sequenced (internal transcribed spacer and 5.8S rDNA). The DNA sequences were submitted to GenBank. Accession numbers are listed in Results and Table 3.

through a fiber wick placed in the bottom of the box as needed.

Collection of *P. tabacina* from field infections. Sixty-six isolates of *P. tabacina* collected from fields in North Carolina (NC), Virginia (VA), Pennsylvania (PA), Connecticut (CT), Kentucky (KY), Georgia (GA), Florida (FL), and Mexico were grown on susceptible tobacco seedlings (cvs. Burley 21 and Bergerac) to increase inoculum (Table 1). The abaxial surface of recipient leaves on healthy plants was inoculated by rubbing infected leaves containing sporangiospores from donor plants. Leaves were sprayed with chilled, sterile distilled water and returned to the sealed plastic boxes. The inoculated seedlings were grown in separate plastic boxes to prevent cross contamination among isolates of *P. tabacina*. The seedlings were grown in a growth chamber at day and night temperatures of 22 and 18ºC, respectively, and under a photoperiod of 10 h of light and 13 h of dark with 1 h of light interruption in the middle of the dark period. Light was provided by a combination of incandescent and fluorescent lights at an intensity of 200 µmol/m^2/s (33).

Cryogenic storage of *P. tabacina* sporangiospores. Sporangiospores from lesions with sporulation were collected 5 days after inoculation. Sporangiospores were harvested by washing the newly sporulating lesions with chilled, sterile distilled water. The resulting suspension was placed in 1.5-ml tubes and centrifuged for 2 min at 12,000 rpm. The supernatant was removed and the procedure repeated to reduce the volume of the suspension. The sporangiospore pellet was transferred to a cryogenic (1.5-ml) tube. A 10% solution of sterile dimethyl sulfoxide (DMSO) was added to each 1.5-ml centrifuge tube and the tubes were placed in a –20ºC freezer overnight and subsequently submersed in liquid nitrogen (–195ºC) for long-term storage (3,5).

Isolates were stored in cryogenic storage and retrieved for subsequent use. Sporangiospores were thawed at room temperature from cryogenic storage and used for subsequent DNA extraction. The DMSO solution was removed with a sterile pipette and the sporangiospores were transferred to a sterile 1.5-ml microcentrifuge tube, washed with sterile distilled water, and centrifuged three times at 12,000 rpm for 2 min.

Culture of other tobacco pathogens. Other important foliar and soilborne fungal pathogens of tobacco were grown on potato dextrose agar (Table 2). Mycelia from cultures in petri dishes were transferred to potato dextrose broth and grown at 25ºC for 3 to 5 days. The potato dextrose broth was filtered and the mycelia were collected. Roughly 100 mg of mycelia were placed in a sterile 1.5-ml microcentrifuge tube for DNA extraction as described below and stored at –20ºC for future use (37).

DNA extraction, PCR, and restriction digests. DNA was extracted from sporangiospores of *P. tabacina* and from mycelia of the other tobacco pathogens. Leaf tissue from healthy tobacco plants served as negative controls. DNA was extracted using the hexadecyltrimethylammonium bromide (CTAB) procedure (36,37). The frozen sporangiospores or mycelia (10 mg) were placed in sterile 1.5-ml microcentrifuge tubes to which 150 µl of extraction buffer (0.35 M sorbital, 0.1 M Tris, 0.005 M EDTA, pH 7.5, and 0.02 M sodium bisulfite) was added, and tubes were vortexed. Nuclei lysis buffer (150 µl; 0.2 M Tris, 0.05 M EDTA, pH 7.5, 2.0 M NaCl, 2% CTAB, and 60 µl of 5% sarkosyl [N-lauryl sarcosine]) was added and tubes were vortexed and incubated at 65ºC for 15 to 30 min. After incubation, one volume (300 µl) of chloroform:isoamyl alcohol (24:1) was added to each tube and tubes were centrifuged for 15 min at 13,000 × *g* at room temperature. The aqueous phase was removed to a new tube and chloroform extraction was repeated. DNA was precipitated overnight at –20ºC in 0.1 volume of 3 M sodium acetate (pH 8.0) and 2 volumes of cold 100% ethanol. The supernatant was discarded; pellets were washed with 70% ethanol and dried under vacuum centrifugation. DNA was suspended in Tris-EDTA (TE; 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). A 1:100 dilution of DNA was made of all extracted DNA for use in PCR experiments.

PCR was conducted in a 50-µl reaction volume in thin-walled 0.2-ml tubes by the procedure of Trout et al. (36). Briefly, 1 µl of template DNA (1:100 dilution of original DNA extract in TE buffer, about 1 ng) was added to a 49-µl master reaction mixture containing 5 µl of 10× PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, and 500 mM KCl, pH 8.3), 35.5 µl of sterile distilled H_2O, 1.8 µl of 10 mM $MgCl_2$, 2 µl of 2 mM dNTPs, 2 µl of 10 µM ITS5 primer, 2 µl of 10 µM ITS4 primer, and 0.2 µl of Taq polymerase (5 U μl^{-1}; Boehriger Mannheim Biochemicals, Indianapolis, IN). The universal fungal primers ITS4 and -5 were used initially to amplify DNA from *P. tabacina* and all the other tobacco pathogens (37). The primer sequences were ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′) and ITS5 (5′-GGAAGTAAA AGTCGTAACAAGG-3′) (38). All reactions were overlaid with sterile mineral oil prior to thermal cycling. Thermal cycling parameters were initial denaturation at 96ºC for 2 min, followed by 35 cycles consisting of denaturation at 96ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 2 min. Thermal cycling was followed by a final extension step at 72ºC for 10 min. Amplified products were separated on 1.6% agarose gels containing ethidium bromide at 0.5 µg/ml with 1× Tris-borate-EDTA (TBE) running buffer. DNA bands were visualized using a UV transilluminator. A 100-bp DNA ladder was included in each gel as a molecular size standard.

Amplified DNA from *P. tabacina* and the other tobacco pathogens were digested with the restriction enzymes *Rsa*I, *Msp*I, *Hae*III, and *Eco*RI to confirm the identity of each species. For each restriction digestion, 1 µl of a master mix consisting of 8 µl of H_2O, 1 µl of restriction enzyme, and 1 µl of the appropriate buffer (React buffer, Gibco BRL, Gaithersburg, MD) was added to 10 µl of PCR product. The reaction mixture was incubated at 37ºC for 3 h and, at the end, restriction enzymes were inactivated by heating at 65ºC for 10 min. The digested products were separated on 1.6% agarose gels containing ethidium bromide at 0.5 µg/ml with 1× TBE running buffer. The RFLP patterns were visualized with a UV transilluminator.

Development of a *P. tabacina*-specific primer. Amplified PCR products (ITS4 and -5 primers) from six isolates of *P. tabacina* (Table 1) and two to three isolates of each of the 12 tobacco pathogens (Table 2) were cleaned using a QIAquick PCR Purification Kit (QIAGEN, Bothell, WA). The amplified fragments were sequenced at the University of Georgia's Molecular Genetics Instrumentation Facility on an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The sequences were aligned utilizing the computer program Clustal W (12). Additional DNA sequences that covered the same amplified regions of DNA from the same species were downloaded from GenBank and used to select the *P. tabacina*-specific primer and to examine restriction sites in each species (Table 2). A region of DNA that was present in *P. tabacina* but absent in the other tobacco pathogens was chosen and a primer was selected and designated PTAB (5′-ATCTTTTTGCTGGCT GGCTA-3′). The primer is located in the first ITS region 106 bases downstream from the universal ribosomal primer ITS5. The PTAB primer was used in conjunction with the primer ITS4 in the *P. tabacina*-specific assay.

The PTAB and ITS4 primers were tested with DNA from 66 isolates of *P. tabacina* (Table 1) and other tobacco pathogens (Table 2). The same PCR protocol was used to test the specificity of the PTAB and ITS4 primers as described above. The PCR product was visualized utilizing 1.5% agarose gels.

Sensitivity of PTAB and ITS4 primers. Extracted DNA from *P. tabacina* was quantified using a spectrophotometer. DNA was diluted from 10 ng ml^{-1} to 0.0125 ng ml^{-1} prior to PCR. Each dilution (1 µl) was used for PCR reactions as described above with primers PTAB and

Table 2. Other tobacco pathogens and *Peronospora* species, isolate number, collector, host, and geographic location of isolates used for polymerase chain reaction and sequencing experiments

Pathogen	Isolate number, GenBank sequence[a]	Collector	Host	Source[b]
Alternaria alternata	1,[c] 2, 3,[a] 4	H. Spurr	Tobacco	NC
Cercospora nicotianae	1, 2, 3,[c] 4	M. Daub	Tobacco	NC
Phytophthora glovera	G-23[c]	D. Shew	Tobacco	Brazil
P. parasitica	326, 371, race 0,[c] race 1	D. Shew	Tobacco	NC
	332, 335	J. Ristaino	Tobacco	NC
Pythium aphanidermatum	L-22,[c] P88	D. Shew	Tobacco	NC
P. dissotocum	P013,[c] P012, Py 47	W. Gutierrez	Tobacco	NC
P. myriotylum	Py-45[c]	W. Gutierrez	Tobacco	NC
P. ultimum	P-74,[c] Py74	D. Shew	Tobacco	NC
Rhizoctonia solani	102[c]	J. Ristaino	Potato	NC
	T-118 T-154, T-73 1708, 19	D. Shew	Tobacco	NC
Sclerotinia sclerotiorum	21, 28, 35,[c] 41	D. Shew	Tobacco	NC
Sclerotium rolfsii	SR-CC-1[c]	J. Ristaino	Tobacco	NC
	SR-DD-8, SR-DD-9	J. Ristaino	Tomato	NC
	SR-DD-5	J. Ristaino	Tomato	AK
Thielaviopsis basicola	1515,[c] 1523	D. Shew	Tobacco	NC

[a] Sequences from GenBank were used to confirm our sequence and restriction site data, including *Alternaria alternata*, AY751456, DQ323699; *Cercospora nicotianae* AF297230, AY266159; *Phytophthora glovera*, AF279126, AF79127, AF279128; *Phytophthora parasitica* (race 0), AY208131, DQ357827; *Pythium aphanidermatum*, AF271227, AB60845, AF271227, DQ298523; *P. dissotocum* AF271228, AF330184; *P. myriotylum*, DQ102701, DQ222438; *P. ultimum,* AY598657, AY310440; *Rhizoctonia solani* AY387569, AB019009; *Sclerotinia sclerotiorum* AB233346, DQ329537; *Sclerotium rolfsii* AB075305, AF499018; *Thielaviopsis basicola* AF275493, U97334; *Peronospora farinosa* AY211017; *P. arenariae*, AY198280; *P. claytoniae,* AY198281; *P. polygoni*, AY198282; *P. holostei*, AY198283; *P. arthurii,* AY198284; *P. boni-henrici,* AY198286; and *P. chenopodii-polyspermi*, AY198291.

[b] NC = North Carolina and AK = Arkansas.

[c] Three to five isolates of each species were sequenced. Sequences from the following isolates (and their accession numbers) were submitted to GenBank: *Alternaria alternata* (1), DQ059568; *Cercospora nicotianae* (3), DQ059569; *Phytophthora glovera* (G-23), DQ059570; *P. parasitica* (race 0), DQ059571; *Pythium aphanidermatum*, (L22), DQ059572; *P. dissotocum* (PO13) DQ059573; *P. myriotylum* (Py-45), DQ059574; *P. ultimum* (P-74), DQ059575; *R. solani* (102) DQ059576; *Sclerotinia sclerotiorum* (35), DQ059577; *Sclerotium rolfsii* (SR-CC-1), DQ059578; and *T. basicola* (1515) DQ059579.

ITS4. The experiment was conducted two times.

Detection of *P. tabacina* from fresh, air-dried, and cured tobacco leaf tissue. Tobacco leaves were inoculated and leaves containing sporulating lesions of *P. tabacina* were harvested as described previously. Leaves either were frozen, air-dried for 2 weeks, or placed in a herbarium drier at 50ºC for 2 weeks to dry lesions and simulate tobacco curing. DNA samples were extracted from lesions containing the pathogen, from symptomless tissue adjacent to lesions, and from other uninfected leaves using the CTAB extraction protocol (36). DNA was diluted 1:100 in TE and 1 µl of template DNA was used in PCR reactions with the PTAB and ITS4 primers as described previously. Amplified products were visualized on 1.5% agarose gels.

RESULTS

DNA was extracted from the sporangiospores of isolates of *P. tabacina* and amplified with the ITS primer pairs ITS4 and ITS5. This region of DNA was sequenced in six isolates of *P. tabacina* and was 862 bp in size (Fig. 1; Table 3). Amplified DNA from all the isolates of *P. tabacina* shown in Table 1 was digested with a series of restriction enzymes. Restriction sites for *Rsa*I and *Hae*III were not found in this region of amplified DNA in any of the isolates of *P. tabacina* tested (Tables 1 and 3). However, restriction sites for *Msp*I and *Eco*RI were found in the amplified rDNA of all the isolates of *P. tabacina* tested, and fragments of 586 and 276 bp for *Msp*I and 540 and 322 bp for *Eco*RI, respectively, were observed (Table 3).

DNA sequences from the ITS region and the 5.8S rDNA were identical among all of the isolates of *P. tabacina* that were sequenced, and the absence of *Rsa*I and *Hae*III and the presence of the *Msp*I and *Eco*RI restriction sites were confirmed (Table 3). The sequence data was submitted to GenBank (*P. tabacina* NC-99-9, DQ059580; BPTP DQ67896; PT 87 Y, DQ067897; NC-99-4, DQ067898; NC-99-6, DQ067899; and Columbia, DQ067900).

The ITS regions and 5.8S rDNA from the other tobacco pathogens also were digested with restriction enzymes *Rsa*I, *Msp*I, *Hae*III, and *Eco*RI (Table 3). Sequence data was used to design the PTAB primer (Table 2). Sequence data from other closely related *Peronospora* spp. were obtained from GenBank (2).

Specificity of the PTAB-specific primer. The PTAB and ITS4 primers were used to amplify rDNA from 66 isolates of *P. tabacina* and isolates of other species that infect tobacco pathogens (Tables 1 and 2). The PTAB primer amplified a 764-bp DNA fragment from *P. tabacina* (Fig. 2). DNA from all the species was amplified with primers ITS4 and -5 prior to PCR, with the primers PTAB and ITS4 as a positive control, and an amplified fragment was obtained for each of the other species (Fig. 1). The PTAB/ITS4 primer pair amplified DNA only from *P. tabacina* and not the other tobacco pathogens shown in Table 2. The PTAB primer annealing site also was present in several other closely related *Peronospora* spp. that are in the same

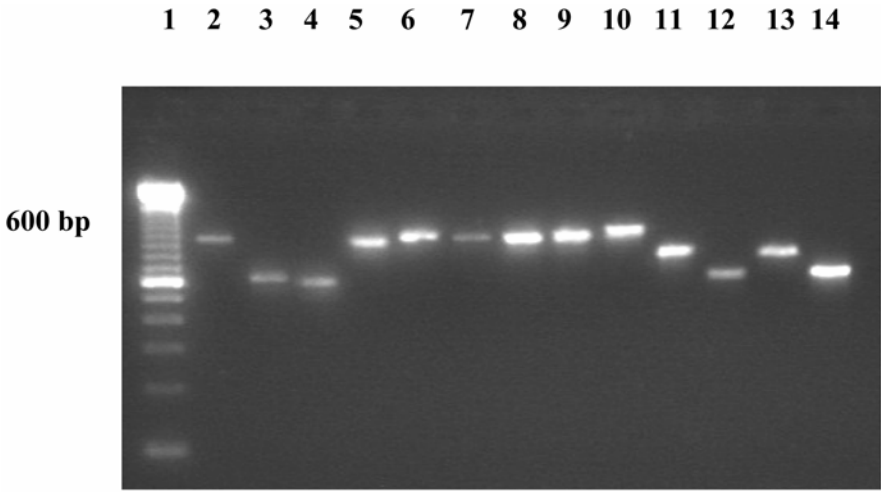

Fig. 1. Polymerase chain reaction-amplified fragments with the primers ITS4 and ITS5 from *Peronospora tabacina* and other tobacco pathogens. Lane 1, 100-bp ladder; lane 2, *P. tabacina* NC-99-9; lane 3, *Alternaria alternata* A4; lane 4, *Cercospora nicotianae* 3; lane 5, *Phytophthora glovera* G-23; lane 6, *P. parasitica* 335; lane 7, *Pythium aphanidermatum* L-22; lane 8, *P. dissotocum* Py-47; lane 9, *P. myriotylum* Py-45; lane 10, *P. ultimum* Py-74; lane 11, *Rhizoctonia solani* 1708; lane 12, *Sclerotinia sclerotiorum*, 21; lane 13, *Sclerotium rolfsii* SR-DD-5; and lane 14, *Thielaviopsis basicola* 1523.

Table 3. Tobacco pathogens, size of internal transcribed spacer (ITS) and 5.8S rDNA region, and restriction fragment sizes with restriction enzymes *Rsa*I, *Msp*I, *Hae*III, or *Eco*RI

	Fragment size (bp)[a]				
Pathogen	**rDNA and ITS amplicon**	***Rsa*I**	***Msp*I**	***Hae*III**	***Eco*RI**
Peronospora tabacina[b]	862	ns	586, 276	ns	540, 322
Alternaria alternata[c]	552	251, 215, 86	412, 146	415, 137	297, 255
Cercospora nicotianae	535	ns	269, 123, 143	339, 88, 77,41	282, 253
Phytophthora glovera	817	342, 274, 106, 85, 10	270, 221, 209, 117	530, 287	ns
P. parasitica	866	521, 345	378, 340, 120,28	667, 169, 30	ns
Pythium aphanidermatum	881	372, 205, 194, 110	ns	622, 259	559, 322
P. dissocticum	833	227, 188, 173, 135, 110	ns	610, 223	551, 282
P. myriotylum	834	361, 190, 173, 110	ns	610, 224	553, 281
P. ultimum	857	384, 227, 136, 110	ns	ns	553, 281
Rhizoctonia solani	690	507, 183	ns	525, 101, 64	339, 351
Sclerotinia sclerotiorum	532	368, 164	319, 213	412, 120	266, 266
Sclerotium rolfsii	694	570, 124	545, 149	ns	ns
Thielaviopsis basicola	554	476, 63	448, 106	385, 169	298, 256

[a] Restriction fragment sizes determined from sequence data and GenBank accessions numbers shown in Table 2; ns signifies that the restriction site was not found in the amplified DNA.

[b] The following isolates of *Peronospora tabacina* were sequenced (ITS and 5.8S rDNA) and the DNA sequences were submitted to GenBank. Accession numbers are: *P. tabacina* isolates NC-99-9, DQ059580; BPTP DQ67896; PT 87 Y, DQ067897; NC-99-4, DQ067898; NC-99-6, DQ067899; and Columbia-PA, DQ067900.

[c] Sequences from the following isolates were submitted to GenBank. Accession numbers are: *Alternaria alternata* (1), DQ059568; *Cercospora nicotianae* (3), DQ059569; *Phytophthora glovera* (G-23), DQ059570; *P. parasitica* (race 0), DQ059571; *Pythium aphanidermatum*, (L22), DQ059572; *P. dissotocum* (PO13) DQ059573; *P. myriotylum* (Py-45), DQ059574; *P. ultimum* (P-74), DQ059575; *Rhizoctonia solani* (102) DQ059576; *Sclerotinia sclerotiorum* (35), DQ059577; *Sclerotium rolfsii* (SR-CC-1), DQ059578; and *Thielaviopsis basicola* (1515) DQ059579.

clade (GenBank accession numbers AY198280 to AY198284, AY198286, AY198291, and AY211017); however, these species do not infect tobacco and have different restriction sites for the suite of enzymes examined in our study and can be separated readily from *P. tabacina* (2; *data not shown*).

Sensitivity of PTAB and ITS4 primers. Detection of *P. tabacina* was positive by gel electrophoresis down to levels of DNA of 0.0125 ng ml^{-1} in repeated experiments (*data not shown*).

PCR of *P. tabacina* from fresh, air-dried, and cured leaf tissue of tobacco. The pathogen was detected consistently using PTAB and ITS4 in fresh lesions from infected leaf material (Fig. 3). The percentage of samples that were positive by PCR detection in known infected leaves was 100% in repeated testing of batches of 10 leaves each. The PTAB/ITS4 primer pair also was used to amplify DNA from infected air-dried and heat-cured tobacco leaves and noninfected healthy control leaves. Host DNA was not amplified by the primers. Pathogen DNA was detected in both air-dried and cured leaf material (Fig. 4). The pathogen also was detected in fresh tissue in symptomless areas adjacent to leaf lesions (Fig. 4).

DISCUSSION

The ability to rapidly and accurately identify pathogens causing plant disease is extremely important in management. We designed a specific primer called PTAB that is located in the spacer one region of the ribosomal DNA for use in the detection of *P. tabacina* in infected tobacco tissue. DNA from *P. tabacina* was successfully amplified from all isolates tested with PTAB and ITS4. The PTAB and ITS4 primers did not amplify DNA from the other common fungal species that infect tobacco or from the tobacco host. Therefore, the primers are specific for *P. tabacina*.

P. tabacina can be differentiated from several different foliar and soilborne tobacco pathogens, including: *Alternaria alternata*, *Cercospora nicotianae*, *Phytophthora glovera*, *P. parasitica*, *Pythium aphanidermatum*, *P. dissotocum*, *P. myriotylum*, *P. ultimum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii,* and *Thielaviopsis basicola*, by PCR-RFLP methods. The enzyme *Rsa*I was the most useful for digestion of DNA amplified from sporangiospores or pure cultures because restriction fragment patterns were unique for most of the pathogens examined, except *P. aphanidermatum* and *P. myriotylum*. The PCR-RFLP assays are useful for identification of isolates that have been obtained in culture. However, because *Peronospora tabacina* is an obligate pathogen, this method is of limited use for diagnosis in infected plant material.

The use of PCR has several advantages over previous diagnostic techniques (16,38). The PTAB and ITS4 primers specifically amplify *P. tabacina* DNA, and this region of DNA is distinct from the other common tobacco pathogens tested. Wiglesworth designed a primer pair (1602A/B) that also specifically amplified *P. tabacina* DNA (38). However, their primer was developed based on RAPD markers and it is unknown what region of the genome of *P. tabacina* is actually amplified with these primers; therefore, they may be subject to genetic variation within populations (32). Also, their primers have

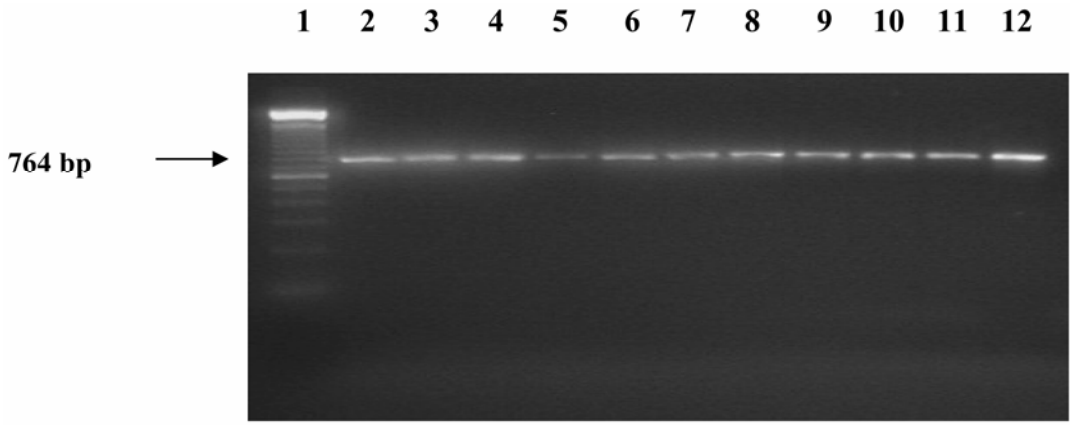

Fig. 2. Polymerase chain reaction amplified DNA with primers PTAB and ITS4 from sporangiospores from 11 different isolates of *Peronospora tabacina*, including lane 1, 100-bp ladder; lane 2, OPT83; lane 3, OPT84; lane 4, PT97H; lane 5, KPT; lane 6, BPTP; lane 7, BPTS; lane 8, JPT84; lane 9, OPT85; lane10, JPT; lane 11, Stokes; lane 12, PT91A.

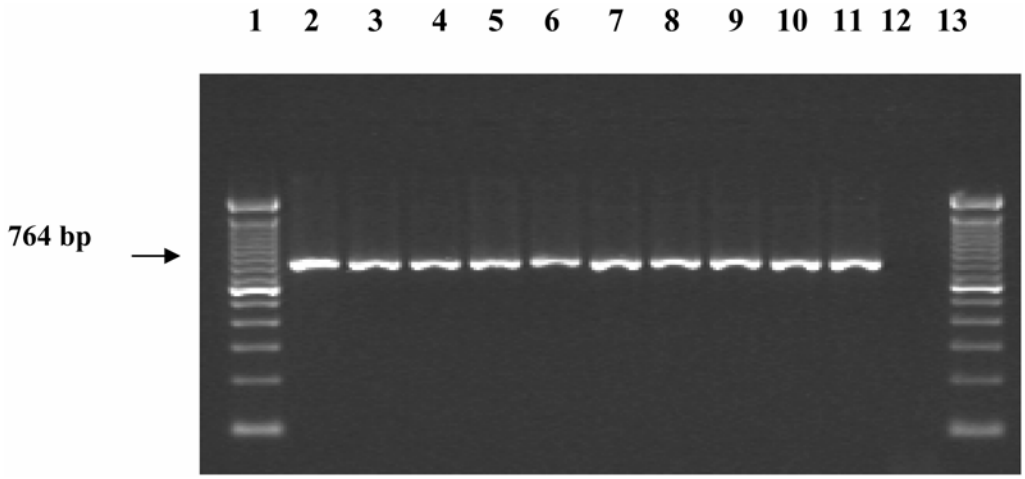

Fig. 3. Detection of *Peronospora tabacina* with primers PTAB and internal transcribed spacer 4 from 10 infected fresh tobacco lesions (lanes 2–11). Non-template control is in lane 12 and lanes 1 and 13 are 100-bp ladders.

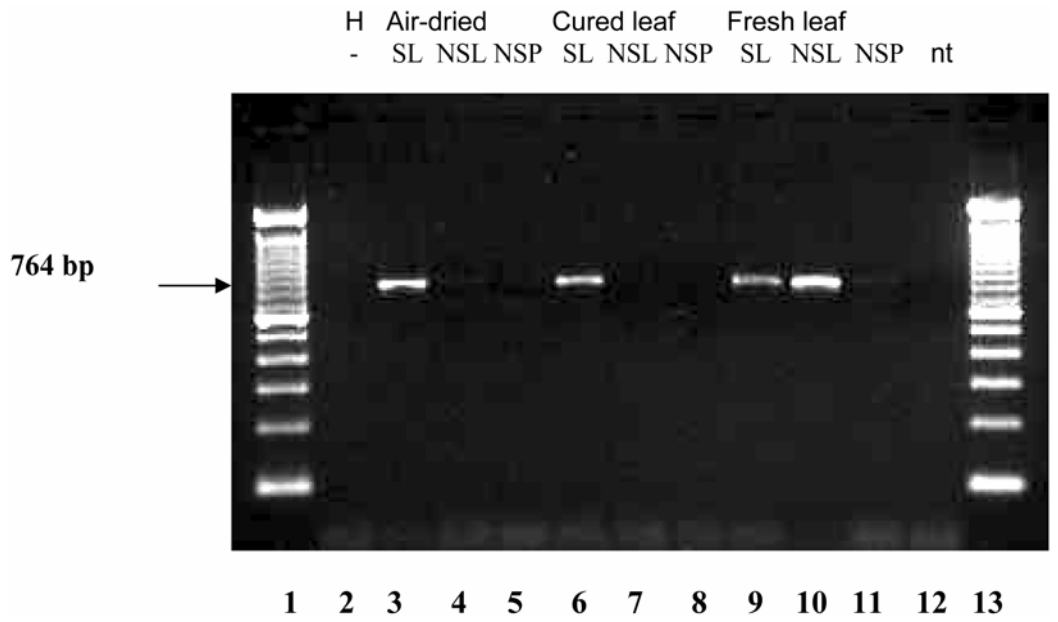

Fig. 4. Amplified DNA from *Peronospora tabacina*-infected tobacco leaves. Polymerase chain reaction products shown are from healthy (H) (lane 2), air-dried (lanes 3–5), flue-cured (6–8), and fresh lesions (9–11) and nontemplate (nt) controls (lane 12). Lanes 1 and 13, 100-bp ladder. Samples were from the symptomatic lesions (SL) (lanes 3, 6, and 9), nonsymptomatic areas adjacent to the lesion (NSL) on the same leaf (lanes 4, 7, and 10) or from a nonsymptomatic leaf (NSP) on the same plant (lanes 5, 8, and 11).

not been tested widely against multiple isolates of *P. tabacina* or other tobacco pathogens (4). The rDNA region we amplified is extremely conserved and has been used for species identification previously in other Oomyceteous and fungal plant pathogens (10,25–27,30). Our primers are more likely to remain useful for species-level diagnostics among many isolates because they worked well with collections of 66 isolates from 1945 to the present.

Other options for pathogen genotyping include RAPDs and RFLP fingerprinting (4,38). RAPDs are useful because they may be able to differentiate different strains of a particular species; however, RAPDs are very sensitive to "nontarget" DNA and may give false positives (39). Sukno developed DNA probes for genotyping *P. tabacina* using RFLP fingerprinting, but the assays were subject to inconsistency due to contaminating bacterial DNA (32). Because our primers are specific for eukaryotic DNA, bacterial contamination was not an issue. The RFLP methods are very time consuming and require large quantities of pure DNA (4,10). Dot blots and reverse dot blots might be useful for pathogen diagnostics (16). Several different pathogens could be tested in a single assay; however, the testing procedure is more complicated than a quick PCR test.

Diagnosis of *P. tabacina* in infected tobacco can be a difficult task. Visual observation of symptomatic sporulating lesions followed by microscopic examination is considered the minimum requirement for positive identification of tobacco blue mold (17,38). In addition, plants with systemic blue mold do not exhibit classical symptoms. The ability to quickly detect the tobacco blue mold pathogen where early signs of infection such as deformed leaves or chlorosis first appear but before sporulation occurs will greatly enhance the ability to identify the disease in transplants. This ability to accurately detect and confirm *P. tabacina* with confidence will aid extension personnel and growers with tough management decisions. This PCR method will allow a quick determination of whether plants are infected with the pathogen and allow diagnosticians to determine the extent of the problem in the field, seed bed, or greenhouse. The decision to spray or not to spray, or even to destroy the crop, can be very costly and these decisions are not made lightly. The PTAB and ITS4 primers could greatly aide the risk-assessment process (39). The PTAB and ITS4 primers were tested and work well in pathogen-infected fresh, air-dried, and cured tobacco tissue and do not amplify host DNA. The primer is very robust in infected host material and consistently yielded positive results in leaves with symptoms.

Tobacco blue mold is a disease of tobacco that has the capability of causing an explosive epidemic under the proper environmental conditions. Because the disease is very common and damaging to tobacco in the southeastern United States, along the eastern seaboard of the United States into Canada, and in Europe, the use of specific primers for the positive diagnosis of *P. tabacina* in infected tobacco could alleviate the necessity of waiting for the visual observation of sporulating lesions. We currently are developing a real-time PCR assay for the pathogen, as has been done with related oomycetes including *Phytophthora ramorum* and other *Peronospora* spp. (2,11). Ideally, an assay that can be deployed to analyze samples in the field should be developed to enable rapid and accurate detection of *P. tabacina* by extension and regulatory personnel.

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