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PCR Primers That Allow Intergeneric Differentiation of Ascomycetes and Their Application to *Verticillium* spp.

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A pair of conserved PCR primers, designated NMS1 and NMS2, that amplify a region in the mitochondrial small rRNA gene region were designed for fungi belonging to the class Ascomycetes. These primers were tested with members of eight fungal genera (*Aspergillus*, *Fusarium*, *Magnaporthe*, *Mycospharella*, *Neurospora*, *Saccharomyces*, *Sclerotinia*, *Verticillium*) and 10 *Verticillium* species (*Verticillium albo-atrum*, *Verticillium chlamydosporium*, *Verticillium cinnebarium*, *Verticillium dahliae*, *Verticillium fungicola*, *Verticillium lecanii*, *Verticillium lateritium*, *Verticillium nigrescens*, *Verticillium psaliotae*, and *Verticillium tricorpus*). The primers were also tested with 35 isolates of *V. dahliae* obtained from diverse geographic areas and diverse hosts. The results of a restriction fragment length polymorphism analysis of the region amplified by the primers differentiated the genera examined and the results of a DNA sequence analysis of the amplified region differentiated the *Verticillium* species. Two *Fusarium* species were also differentiated by the results of the restriction fragment length polymorphism analysis. On the basis of the nucleotide sequences of the amplified regions, we obtained a pair of PCR primers that could be used to differentiate *V. dahliae* from the other fungal isolates tested, including *V. albo-atrum*, a closely related plant-pathogenic species. The *V. dahliae*-specific PCR primer may aid in more rapid and specific detection of the pathogen directly in plant and/or soil samples. PCR primers NMS1 and NMS2 may be used as potential mitochondrial markers for studying fungal cytoplasmic inheritance of ascomycetes and for identifying DNA probes that are informative at or below the genus level.

The form genus *Verticillium* contains two economically important plant pathogens, *Verticillium dahliae* Kleb. and *Verticillium albo-atrum* Reinke et Berthold (16, 23, 25). These organisms are detected and the levels of *Verticillium* inocula are estimated by dilution plating on semiselective media or by similar procedures (3). These methods are labor intensive and require 2 to 4 weeks to complete. In addition, results are variable, and false-negative results occur because of dormancy of microsclerotia or inhibition of growth by antagonists that are not suppressed by the selective medium. Numerous attempts have been made to develop specific antisera for *Verticillium* pathogens (4–6, 8, 17, 18, 29, 31). None of the antisera developed has been widely used to discriminate between *V. dahliae* and *V. albo-atrum*.

Rapid and sensitive assay methods in which molecular techniques are used are becoming important tools for detecting microorganisms in the environment (24). Nazar et al. (19) described a pair of PCR primers that can be used for specific detection of *V. dahliae* and *V. albo-atrum*. These primers were tested with three *V. dahliae* isolates and four *V. albo-atrum* isolates and were subsequently used to detect *V. albo-atrum* in plant samples (9).

Nuclear and mitochondrial ribosomal genes have been used as molecular probes because they represent a wide range of rates of evolution and levels of divergence and hence allow differentiation and detection at a wide range of specificities (22). Mitochondrial DNA is known to have a higher rate of evolution than nuclear DNA (1), so it has greater potential for use as a tool to differentiate closely related species. White et al. (30) described a pair of PCR primers that amplified a region of the mitochondrial rRNA gene in several fungi. The design of these primers was based on the sequence of a basidiomycete, *Suillus sinuspaulianus*. These primers did not work consistently for some ascomycetes (14).

We have developed a pair of primers that amplify a portion of the mitochondrial small rRNA gene in ascomycetes (13, 15). In this paper we provide evidence that the amplified region has sufficient heterogeneity to allow differentiation among genera of ascomycetes and among *Verticillium* and *Fusarium* species. Using the sequence data for this gene, we developed a pair of primers that amplified a 140-bp region of *V. dahliae* DNA but no other DNA tested.

MATERIALS AND METHODS

Fungal isolates. The origins, identities, and designations of the fungal isolates used in this study are shown in Table 1. The *Verticillium* isolates used were derived from single conidial spores produced on potato dextrose agar. All single-spore cultures were examined microscopically to confirm their identities.

Genomic DNA extraction. Isolates were grown on potato dextrose agar at room temperature for 3 to 7 days. Agar plugs (diameter, 0.5 cm) were cut from the actively growing edges of the colonies and were inoculated into two 250-ml flasks containing 100 ml of potato dextrose broth. After 3 to 7 days of incubation with constant shaking, mycelium was harvested by filtering each preparation through Whatman no. 1 filter paper, rinsed with sterile distilled water, and lyophilized, and 0.2 g of the lyophilized mycelium was ground in liquid N_2, suspended in 4 ml of extraction buffer (50 mM Tris-Cl [pH 8.0], 850 mM NaCl, 100 mM EDTA, 1% sodium dodecyl sulfate [SDS]), and thoroughly mixed; 0.4 ml of 10% CTAB (hexadecyltrimethylammonium bromide) in 0.7 M NaCl was then added. The mixture was then incubated at 65°C for 15 min and extracted with 0.5 volume of Tris-HCl-saturated phenol (pH 8.0), 0.5 volume of chloroform-isoamyl alcohol (24:1), and then 1

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TABLE 1. Designations, identities, geographic origins, host origins, and sources of fungal isolates

Isolate	Species	Geographic location[a]	Host or origin[a]	Source[b]
V18	*Verticillium dahliae*			D. I. Rouse
V14	*V. dahliae*	Wisconsin	Potato	D. I. Rouse
V1654	*Verticillium* sp.[c]	Wisconsin	Poppy	D. I. Rouse
V1665	*V. dahliae*	Wisconsin	Potato	D. I. Rouse
LV64	*V. dahliae*	Wisconsin	Potato	K.-N. Li
LV75	*V. dahliae*	Oregon		D. I. Rouse
Mint (UMD-7)	*V. dahliae*	Michigan	Peppermint	D. I. Rouse
B111	*V. dahliae*	Syria	Cotton	A. Bell
B207	*V. dahliae*	Australia	Cotton	A. Bell
SZ-1 (CS-1)	*V. dahliae*	Swaziland	Cotton	A. Bell
CW (49)	*V. dahliae*	Washington	Cherry	A. Bell
FN (37)	*V. dahliae*	Australia	Flax	A. Bell
MC (79)	*V. dahliae*	California	Chrysanthemum	A. Bell
MG (56)	*V. dahliae*	Indiana	Mint	A. Bell
PCW (124)	*V. dahliae*	California	Pepper	A. Bell
P-1-1	*V. dahliae*	California	Cotton	A. Bell
P-1-3	*V. dahliae*	California	Olive	A. Bell
P-1-4	*V. dahliae*	Arkansas	Cotton	A. Bell
P-2-1	*V. dahliae*	Italy	Eggplant	A. Bell
P-2-7	*V. dahliae*	Canada	Watermelon	A. Bell
227 (19)	*V. dahliae*	Washington	Sugar beet	A. Bell
BB (21)	*V. dahliae*	Idaho	Potato	A. Bell
FN (37)	*V. dahliae*	Australia	Flax	A. Bell
PS (39)	*V. dahliae*	Australia	Pelargonium	A. Bell
VW (50)	*V. dahliae*	Wisconsin	Velvetleaf	A. Bell
EN (97)	*V. dahliae*	The Netherlands	Eggplant	A. Bell
S52	*V. dahliae*	Ohio	Potato	R. Rowe
S70	*V. dahliae*	Ohio	Potato	R. Rowe
TO (120)	*V. dahliae*	Canada	Tomato	A. Bell
V25	*V. dahliae*	Idaho	Potato	R. Rowe
81-38A	*V. dahliae*	California	Guayule	R. Rowe
NRRL 13687	*V. dahliae*		(= CMI45492)	K. O'Donnell
NRRL 1204	*V. albo-atrum*			K. O'Donnell
LV79	*V. albo-atrum*	Wisconsin	Alfalfa	C. Grau
Superior	*V. albo-atrum*	Wisconsin	Potato	D. I. Rouse
DV20	*V. albo-atrum*	Idaho		J. Davis
Tricorpus	*V. tricorpus*			J. Davis
DV32	*V. tricorpus*			J. Davis
DV52	*V. tricorpus*			J. Davis
NRRL 13690	*V. tricorpus*		(= CMI238594)	K. O'Donnell
Nigrescens	*V. nigrescens*			D. I. Rouse
NRRL 13093	*V. chlamydosporium*		(= ATCC 52033)	K. O'Donnell
NRRL A-3665	*V. cinnebarium*			K. O'Donnell
NRRL 13900	*V. fungicola*			K. O'Donnell
NRRL A-18693	*V. lateritium*			K. O'Donnell
NRRL A-18240	*V. lecanii*		(= CBS297.64)	K. O'Donnell
NRRL A-18376	*V. psaleotae*			K. O'Donnell
Fusarium sp.	*Fusarium* sp.			P. Williams
A149	*F. moniliforme*	California	Maize	E. Smalley
B3852	*F. subglutinans*		Laboratory cross[d]	E. Smalley
D4853	*F. proliferatum*		Laboratory cross[e]	E. Smalley
F4092	*F. moniliforme*		Laboratory cross[d]	E. Smalley
F4093	*F. moniliforme*		Laboratory cross[d]	E. Smalley
FO1	*F. oxysporum*			E. Smalley
FO23D	*F. oxysporum*			E. Smalley
FO119	*F. oxysporum*			E. Smalley
FO916	*F. oxysporum*		Barley grains	R. Caldwell
FO1042	*F. oxysporum*		Date palms	R. Caldwell
FO1055	*F. oxysporum*		Carrot	R. Caldwell
Aspergillus	*Aspergillus parasiticus*			J. Andrews
Leptosphaeria	*Leptosphaeria macubus*			S. Leong
Magnoporthe	*Magnaporthe grisea*		Rice	A. Ellingboe
Neurospora	*Neurospora crassa*			G. H. Feng
Sclerotinia	*Sclerotinia sclerotiorum*	Wisconsin	Snap beans	W. Stevenson
Ustilago	*Ustilago maize*			S. Leong

[a] No information means that the origin is not known. ATCC, American Type Culture Collection, Rockville, Md.; CMI, Commonwealth Mycological Institute, Kew, England; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

[b] J. Andrews, R. Caldwell, A. Ellingboe, C. Grau, S. Leong, K.-N. Li, D. I. Rouse, E. Smalley, W. Stevenson, and P. Williams are at the Department of Plant Pathology, University of Wisconsin, Madison; G. H. Feng is at the Department of Genetics, University of Wisconsin, Madison; A. Bell is at the Department of Plant Pathology, Texas A & M University, College Station; K. O'Donnell is at the Midwest Area National Center for Agricultural Utilization Research, USDA/ARS, Peoria, Ill.; R. Rowe is at the Department of Plant Pathology, Ohio State University, Wooster; and J. Davis is at the Department of Plant Pathology, University of Idaho, Aberdeen.

[c] Not identified.

[d] Origins are described in reference 11.

[e] See reference 11a.

volume of chloroform-isoamyl alcohol. The DNA was then precipitated with 2.5 volumes of 100% ethanol, rinsed with ice-cold 70% ethanol, and dissolved in 4 ml of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]) containing RNase Plus (5 Prime → 3 Prime, Inc., Boulder, Colo.). The quality of the DNA was determined, and the DNA concentration was determined by agarose gel electrophoresis and by spectrophotometry. All DNA extracts that were going to be used as PCR templates were diluted to a final concentration of 10 ng/μl.

PCR. Unless indicated otherwise, the PCR was performed in 25 μl (total volume) of a solution containing 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dTTP, 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, and 0.1% Triton X-100; the mixture was prepared with ice-cold stock solutions and was kept on ice until the reaction tubes were put onto a preheated hot block kept at 95°C. The initial cycle consisted of 95°C for 3 min, the annealing temperature for 1 min, and 72°C for 1 min; this was followed by 34 cycles consisting of 95°C for 1 min, the annealing temperature for 1 min, and 72°C for 1 min. The final step was a 4-min chain extension step at 72°C. The PCR were performed with a programmable DNA thermocycler (Coy Corp., Grass Lake, Mich.), a TwinBlock Systems apparatus (Ericomp, Inc., San Diego, Calif.), or a model 480 DNA thermal cycler (Perkin-Elmer Corp., Norwalk, Conn.). The annealing temperatures were different for different PCR primers. The PCR products were analyzed by electrophoresis on 1% agarose gels in 0.5× TBE buffer (1× TBE buffer is 0.090 M Tris, 0.090 M boric acid, and 2 mM EDTA [pH 8.0]) containing 10 ng of ethidium bromide per ml or on 6% polyacrylamide gels in 1× TBE buffer which were stained with ethidium bromide and visualized with a UV transilluminator (Fotodyne, Inc., Hartland, Wis.).

Restriction digestion of the PCR products. PCR products were cleaned by phenol-chloroform extraction and ethanol precipitation. The cleaned products were quantified with a minifluorometer (model TKO 100; Hoefer Scientific, San Francisco, Calif.) and were digested with a restriction endonuclease as recommended by the manufacturer. The digestion products were analyzed by electrophoresis on agarose or polyacrylamide gels as described above.

Cloning of PCR products. The PCR products obtained from isolates V18, V14, DV20, Superior, and Tricorpus, which represented the three plant-associated species of the genus *Verticillium* (Table 1), were amplified with primers NMS1 and NMS2 and were cloned into plasmid pCR1000 with a TA cloning kit (Invitrogen, San Diego, Calif.) by following manufacturer's protocol.

DNA sequencing and sequence analysis. For direct PCR sequencing, the PCR products were electrophoresed on a 1% agarose gel. The gel slice containing the desired PCR products was cut out and rinsed with TE buffer, and the DNA was extracted and purified by using a Prep-A-Gene DNA purification kit (Bio-Rad, Richmond, Calif.) as recommended by the manufacturer. The concentration of the purified DNA in TE buffer was determined by using a fluorometer as described above, and 20 to 200 ng of DNA was sequenced by using the *fmol* DNA sequencing system (Promega Corp., Madison, Wis.) as recommended by the manufacturer. To sequence cloned PCR products, 500 to 1,000 ng of miniprepped plasmid DNA (26) from plasmid pCR1000 containing the desired PCR products was sequenced with the *fmol* system or with a Sequenase DNA sequencing kit (United States Biochemicals Corp., Cleveland, Ohio). Two clones of each of the isolates were sequenced completely to minimize errors. The DNA sequences were analyzed with the DNA analysis software package of the Genetic Computing Group (7).

Southern hybridization. (i) Gel electrophoresis and Southern transfer. PCR products were analyzed on a 1% agarose gel and were photographed with a UV transilluminator. The gel was then denatured in 1.5 M NaCl–0.5 M NaOH for 30 min, neutralized in 3 M NaCl–0.5 M Tris-HCl (pH 7.0) for 30 min, and blotted onto a Zeta-Probe blotting membrane (Bio-Rad) by using the standard Southern blotting protocol provided by the manufacturer. After the transfer was complete (4 h to overnight), the DNA was cross-linked to the nylon membrane by exposing the membrane to UV light with a UV transilluminator (Fotodyne) for 5 min.

(ii) Probe labeling. When the PCR products were used as the probe, they were first purified by using agarose electrophoresis and a Prep-A-Gene cleaning kit as described above. Then 50 to 200 ng of DNA was labeled with [^{32}P]dCTP (Amersham, Arlington Heights, Ill.) by using a random priming kit (Boehringer Mannheim, Mannheim, Germany) as recommended by the supplier. The oligonucleotide probe was labeled with terminal transferase (Promega) by using a [^{32}P]dCTP/probe molar ratio of approximately 10:1. The labeled probes were separated from the unincorporated nucleotides by using Sepharose 50 spin columns (26).

Hybridization and subsequent washing procedures were performed by using type HD-1D hybridizer (Techne, Inc., Princeton, N.J.). For the PCR product probe, hybridization and washing were done under high-stringency conditions (0.1× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA; pH 7.7] containing 0.1% SDS, 65°C, 5 h). For the oligonucleotide probe, the following temperature conditions were used: hybridization at 42°C overnight and washing three times (30 min each) at 50°C. When it was necessary, the hybridized membrane was stripped of radioactive probes by incubating it in 0.1× SSPE–0.1% SDS at 95°C overnight so that it could be used with another probe.

PCR primer design. The PCR primers which we designed had G+C contents of approximately 50 mol% and were 21 to 24 bases long. When PCR primers were designed to distinguish between organisms that had only a single base difference, the mismatched base was placed at the last position at the 3′ end of the primer. The chain extension reaction was least efficient in such a case; this technique maximized the effects of the mismatch and resulted in increased specificity for distinguishing the organisms. The PCR primers were synthesized by workers at the University of Wisconsin Biotechnology Center.

Nucleotide sequence accession numbers. The GenBank accession numbers of the nucleotide sequences used in this study are as follows: *Aspergillus nidulans* MS1 locus, V00653; *Neurospora crassa* MS1 locus, J05254; *Penicillium chrysogenum* MS1 locus, L01493; *Podospora anserina* MS1 and MS2 loci, X55026; *Saccharomyces cerevisiae* MS1 and MS2 loci, X14966; *Schizosaccharomyces pombe* MS1 and MS2 loci, X54421; *Aspergillus nidulans* MS2 locus, J01393; *Penicillium chrysogenum* MS2 locus, Z23092; *Schizosaccharomyces japonicus* MS2 locus, X72084; *V. dahliae*, L18970; *V. albo-atrum*, L18971.

RESULTS

Inconsistent PCR results obtained with original primers MS1 and MS2. When primers MS1 and MS2 (30) were used in the PCR to amplify the intended region of the mitochondrial small rRNA subunit gene in *Verticillium* strains, inconsistent amplification results occurred at an annealing temperature of 55°C. We found that consistent amplification results were obtained only when the annealing temperature was decreased to 35°C (14) (Fig. 1). This temperature sensitivity could be explained by the occurrence of substantial mismatches be-

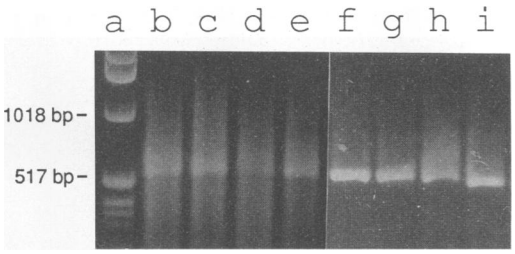

FIG. 1. Agarose gel electrophoresis of PCR results obtained with primers MS1 and MS2 and isolate V18 genomic DNA by using different annealing temperatures and two Mg^{2+} concentrations. Lane a, Bethesda Research Laboratories 1-kb molecular weight marker; lane b, annealing temperature of 55°C, 2.5 mM Mg^{2+}; lane c, annealing temperature of 55°C, 5.0 mM Mg^{2+}; lane d, annealing temperature of 50°C, 5.0 mM Mg^{2+}; lane e, annealing temperature of 50°C, 2.5 mM Mg^{2+}; lane f, annealing temperature of 35°C, 5.0 mM Mg^{2+}; lane g, annealing temperature of 35°C, 2.5 mM Mg^{2+}; lane h, annealing temperature of 37°C, 5.0 mM Mg^{2+}; lane i, annealing temperature of 37°C, 5.0 mM Mg^{2+}.

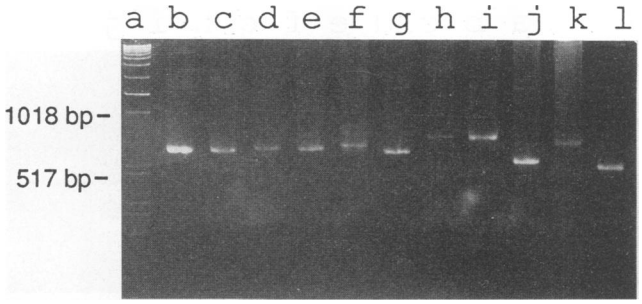

FIG. 3. Polyacrylamide gel electrophoresis gel showing the size variation of the PCR products obtained with primers NMS1 and NMS2 for several ascomycetes. Lane a, Bethesda Research Laboratories 1-kb molecular weight marker; lane b, strain V18; lane c, strain V1654; lane d, strain V1665; lane e, strain V14; lane f, strain Tricorpus; lane g, strain Nigrescens; lane h, *Fusarium* sp.; lane i, *Aspergillus nidulans*; lane j, *Magnaporthe grisea*; lane k, *Neurospora crassa*; lane l, *Ustilago* sp. (a basidiomycete).

tween the primers and the putative binding sites. Cloning and sequencing of the PCR products obtained by using primers MS1 and MS2 revealed that these products were heterogeneous even though they were the same size (14), possibly because the *Taq* DNA polymerase has a high error rate at low primer annealing temperatures (28).

Alignment of previously described GenBank ascomycete sequences at this locus confirmed that there were substantial mismatches between the original MS1-MS2 sequences and the previously described sequences at the locus in ascomycetes. We designed two new primers, designated NMS1 and NMS2, on the basis of the previously described GenBank sequences for the mitochondrial small rRNA genes of ascomycetes (Fig. 2). The new primers resulted in much improved PCR results (Fig. 3).

Variability of the amplified region. Using the new primers, we amplified this region from members of eight fungal genera, members of nine *Verticillium* species, 35 isolates of *V. dahliae*, and 11 *Fusarium* isolates representing four species (Table 1).

a

Source	Sequence
NMS1	CAGCAGTGAGGAATATTGGTCAATG
MS1	CAGCAGTCAAGAATATTAGTCAATG
Aspergillus nidulans	CAGCAGTGAGGAATATTGGTCAATG
Neurospora crassa	CAGCAGTGAGGAATCTTGGTCAATG
Penicillium chrysogenum	CAGCAGTGAGGAATATTGGTCAATG
Podospora anserina	CAGCAGTGAGGAATATTGGTCAATG
Saccharomyces cerevisiae	CAGCAGTGAGGAATATTGGACAATG
Schizosaccharomyces pombe	CAGCAGTGGAGAATATTAGTCAATG

b

Source	Sequence
NMS2	GCGGATCATCGAATTAAATAACAT
MS2	GCGGATTATCGAATTAAATAAC..
Aspergillus nidulans	GCGGATCATCGAATTAAATAACAT
Penicillium chrysogenum	GCGGATCATCGAATTAAATAACAT
Podospora anserina	GTGGGTCATCGAATTAAATAACAT
Saccharomyces cerevisiae	GTGGATTATCGAATTAAATAACAT
Schizosaccharomyces japonicus	GTGGATTATCGAATTAAATAACAT
Schizosaccharomyces pombe	GCGGATTATCTAATTAAATAACAT

FIG. 2. (a) Comparison of the nucleotide sequences of the MS1 locus from several ascomycetes. MS1 was the original primer, and NMS1 was the modified primer. (b) Comparison of the nucleotide sequences of the MS2 locus from several ascomycetes. MS2 was the original primer, and NMS2 was the modified primer.

Some of the PCR results are shown in Fig. 3; the data illustrate the length polymorphism at this locus at the genus level.

The variability within the amplified region was also investigated by restriction digestion of the PCR products with *Mse*I. The restriction fragment length polymorphism (RFLP) patterns for this region differed not only at the generic level but also among *Verticillium* species (Fig. 4).

When the whole PCR fragment from *V. dahliae* was used as the probe, Southern hybridization revealed that *V. dahliae*, *V. albo-atrum*, and *Verticillium tricorpus*, all of which are known to be associated with plants, could be easily distinguished from all of the other *Verticillium* species and other fungal groups tested, including the *Fusarium* species (Fig. 4). When an oligonucleotide based on the sequence of the amplified region was used as the probe (Fig. 5), we were able to further differentiate *V. tricorpus* from *V. dahliae* and *V. albo-atrum* (Fig. 5C). When the amplified region from *Verticillium lateritium* was used to probe the same blot, it hybridized not only with itself but also with the region from *Verticillium cinnebarium* (Fig. 5D).

The RFLP pattern of the region from members of the genus *Fusarium*, another anamorphic fungus genus whose members include many important plant-pathogenic species, was also different at the intrageneric level (Fig. 4). Five *Fusarium moniliforme* isolates, representing mating populations A, B, D, and F, and six *Fusarium oxysporum* isolates were included in the study. Members of *F. moniliforme* mating populations A, B, and D and four *F. oxysporum* isolates produced the same RFLP pattern, while the *F. moniliforme* mating population F strains and the other two *F. oxysporum* isolates produced their own distinct patterns (Fig. 4).

Further differentiation of *Verticillium* species. The region amplified by NMS1 and NMS2 from *V. dahliae*, *V. albo-atrum*, and *V. tricorpus* was cloned into pCR1000 and sequenced. Sequence data are shown in Fig. 6. A comparison of the sequences revealed a two-base difference. On the basis of this difference, we designed two more PCR primers, designated VMSP1 and VMSP2. These primers differentiated *V. dahliae* from the other two *Verticillium* species under the following conditions: a reaction mixture containing 10 ng of target DNA, 10 pmol of each primer, 20 mM Tris-HCl (pH 8.3), 0.75 mM $MgCl_2$, 25 mM KCl, each deoxynucleoside triphosphate at a concentration of 20 μM, and 0.75 U of *Taq* DNA polymerase in a total volume of 50 μl; and a temperature profile consisting of 94°C for 1 min and 72°C for 2 min for a total of 40 cycles

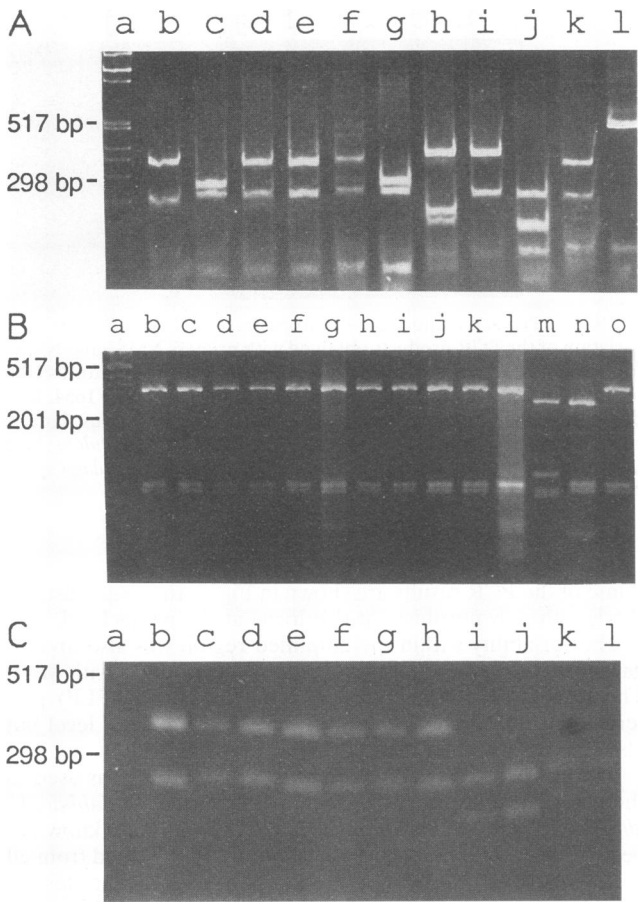

FIG. 4. (A) Polyacrylamide gel electrophoresis gel of the RFLPs of the NMS1-NMS2-amplified regions from selected ascomycetes showing divergence at or below the genus level. Lane a, Bethesda Research Laboratories 1-kb molecular weight marker; lane b, strain V18; lane c, strain V1654; lane d, strain V1665; lane e, strain V14; lane f, strain Tricorpus; lane g, strain Nigrescens; lane h, *Fusarium* sp.; lane i, *Aspergillus nidulans*; lane j, *Magnaporthe grisea*; lane k, *Neurospora crassa*; lane l, *Ustilago* sp. (a basidiomycete). (B) Agarose gel electrophoresis gel of the RFLPs of the NMS1-NMS2-amplified regions from members of three plant-associated *Verticillium* species. Lane a, Bethesda Research Laboratories 1-kb molecular weight marker; lane b, strain CC64; lane c, strain TO; lane d, strain OV25; lane e, strain S70; lane f, strain 81-38A; lane g, strain CW; lane h, strain PCW; lane i, strain P1; lane j, strain S52; lane k, strain DV32; lane l, strain DV52; lane m, strain DV20; lane n, strain Superior; lane o, strain LV75. (C) Agarose gel electrophoresis gel of the RFLPs of the NMS1-NMS2-amplified regions from a selected group of *Fusarium* spp. Lane a, Bethesda Research Laboratories 1-kb DNA molecular weight marker; lane b, strain A149; lane c, strain B3852; lane d, strain D4853; lane e, strain FO1042; lane f, strain FO1055; lane g, strain FO916; lane h, strain FO1; lane i, strain FO119; lane j, strain FO23D; lane k, strain F4093; lane l, strain F4092.

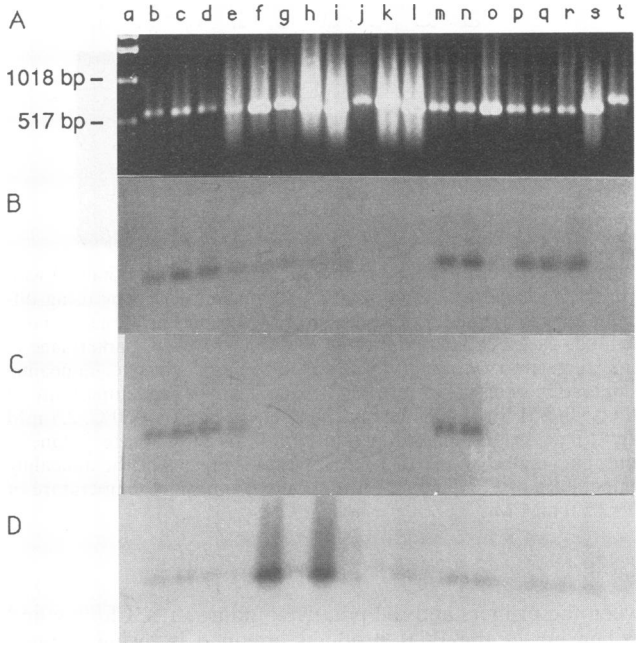

FIG. 5. Agarose gel electrophoresis gel of PCR products from various *Verticillium* spp. amplified with primers NMS1 and NMS2 (A) and Southern hybridization of the same gel by using the labeled whole amplified region from *V. dahliae* (B), part of the region as a probe (Fig. 6) to distinguish the two plant-pathogenic species from the rest of the isolates (C), and the whole amplified region from *V. lateritium* (D). Lane a, Bethesda Research Laboratories 1-kb molecular weight marker; lane b, strain V18; lane c, strain 81-38A; lane d, strain V14; lane e, strain NRRL 13687; lane f, strain NRRL A-18693; lane g, strain NRRL A-18240; lane h, strain NRRL A-3665; lane i, strain NRRL A-18367; lane j, strain NRRL 13093; lane k, strain NRRL 13900; lane l, strain Nigrescens; lane m, strain Superior; lane n, strain NRRL 1204; lane o, strain DV20; lane p, strain NRRL 13690; lane q, strain DV32; lane r, strain Tricorpus; lane s, strain SSB; lane t, *Fusarium* sp.

(Fig. 7). Primers VMSP1 and VMSP2 amplified a 140-bp region from 35 *V. dahliae* isolates. These primers did not amplify the DNAs of six *V. albo-atrum* isolates, the DNAs of five *V. tricorpus* isolates, any other fungal DNA tested, or potato DNA (Fig. 7).

DISCUSSION

We designed a pair of primers, NMS1 and NMS2, that consistently and specifically amplified an approximately 600-bp region in the mitochondrial small rRNA gene of members of eight fungal genera, members of nine *Verticillium* species, and 35 isolates of *V. dahliae* obtained from diverse geographic areas and hosts. Although the priming sites of this mitochondrial locus are conserved among ascomycetes, our results indicated that the region between the sites allowed differentiation of the fungi tested at the intergeneric level, as shown by the RFLP and Southern hybridization results. This region could also be used to differentiate some species within the genera *Verticillium* and *Fusarium*.

Another pair of primers, designated MS1 and MS2, was previously designed for the same locus (30). Our results showed that MS1 and MS2 were not optimal for *Verticillium* isolates and other ascomycetes because of mismatches between the primers and the intended primer binding sites.

Differentiation of *Verticillium* species. The results of Southern hybridization of the amplified region indicated that the three plant-associated fungi, *V. dahliae*, *V. albo-atrum*, and *V. tricorpus*, can be easily distinguished from other *Verticillium* species and members of the other fungal groups tested, including two *Fusarium* species. Our results confirm the current separate taxonomic status of *V. dahliae* and *V. albo-atrum* (10) given the uniformity at this locus found in *V. dahliae* isolates collected from diverse hosts and locations compared with *V. albo-atrum*. The results of Southern hybridization in which the amplified mitochondrial small-subunit rRNA gene

```
  1  GCGGATCATC GAATTAAATA ACATACTTCA CTACTGGTGT CAGAAACGGT
 51  CTAGTGATTT CAGTTCCTGC GTTGCCACAT TACTCTTGAG GTGGAATGCT
101  TTTATTTTCA TTGATAGACT AAATTCTCAT TTAATCTACG ACATTCATCA
151  TTGTCTGCAA AGACTACTGG GGTGTCAAAT CCTGTTTGCG ACCTATGCCT
                          VMSP1                  *
201  TCGTCCTTCA ACGTCAGTTT TTACATAAAA GACTGCCTAC GCCGTTGCCA
251  GTCCCTTTGG TATCACAGAA TTTAATCTCT CCAACTCTAA GTACTGTCTT
                                                    #
301  CTTACATAAA ACTCTAGACA AATATTACCT ATTAAAGGAT AATCTGACCG
           VMSP2
351  TTTGAGTACC CTTTAAACCT ATTTAAGATG AATAACGCTA GTCTCATACG
401  TATTACCGCG ACTGCTGGCA CGTATTTTGT CAAGACCGTA TATACATATC
451  GTCATTATAT CAATGTATTT AAAATTTTAT TCGATTAATC AATTAAATTT
501  TACTTATATT TCTATTTTCA AACTTCCCAT TCTTTCAAGT TGCCAGTTCG
551  GCCGTTAGGC CATTGACTAA TATTCTTGAC TGCTG
```

FIG. 6. Nucleotide sequence of the region of the small subunit of the mitochondrial rRNA gene PCR amplified by primers NMS1 and NMS2. The regions in boldface type are primers VMSP1 and VMSP2, which can be used to differentiate *V. dahliae*, *V. albo-atrum*, and *V. tricorpus*. The region between positions 231 and 260 was used as the oligonucleotide probe to differentiate the two plant-pathogenic species, *V. dahliae* and *V. albo-atrum*, from the rest of the fungal isolates (Fig. 5). The nucleotide at the position marked with an asterisk is G in *V. dahliae* and A in *V. albo-atrum* and *V. tricorpus*. The nucleotide at the position marked with a number sign is G in *V. dahliae* and *V. albo-atrum* and A in *V. tricorpus*.

region from *V. lateritium* was used as the probe supported the conclusion that *V. lateritium* and *V. cinnebarium* are synonyms (10).

Differentiation of *Fusarium* species. Six mating populations (mating populations A through F) have been identified in the *Liseola* section of the genus *Fusarium* (11). The perfect stage of all of the isolates belonging to this section is *Gibberella fujikuroi* (Sawada) Ito. Traditionally, all of these organisms have been placed in one species, *F. moniliforme* (27), but Nirenberg (21) proposed that they should be classified in six species. According to Nelson et al. (20), members of these mating populations belong to different species; members of mating populations B and E belong to *Fusarium subglutinans*, members of mating population D belong to *Fusarium proliferatum*, and members of mating populations A and F belong to *F. moniliforme*, as determined by morphological characteristics and host preferences. Another species, *F. oxysporum*, is closely related but differs in many morphological characteristics, including conidial chain format, presence of chlamydospores, and organization of philiades (20). Our results indicated that, for the amplified mitochondrial rRNA locus, members of mating populations A, B, and D of the *Liseola* section and three *F. oxysporum* isolates produced identical RFLP patterns, while members of mating population F and two other isolates produced distinct patterns (Fig. 4). In a recent study, Leslie et al. (12) found that although members of mating populations A and F have the same anamorph, *F. moniliforme*, their levels of mycotoxin production are significantly different.

Potential use as an assay tool. An immediate use of the species-specific DNA probe would be to augment the dilution plating technique currently used to detect and quantify *Verticillium* pathogens in soil. Accurate and timely identification of pathogens and quantification of the pathogen levels in the environment are important for developing improved procedures for disease control (25). The methods used previously for *Verticillium* isolates require at least 10 days so that sufficient fungal growth can occur on the semiselective media. Questions regarding proper identification of nontypical colonies on plates often arise (3). Identification of *Verticillium* isolates on semiselective medium is based solely on the morphology of the microsclerotial colonies. Mycological confirmation of the identities of seemingly abnormal colonies may be difficult if not impossible, because other fungal growth on the plate induces abnormal colony morphology of *Verticillium* isolates and also interferes with the purification of colonies. Thus, in some cases, the counting results obtained by the dilution plating technique may be inaccurate (3).

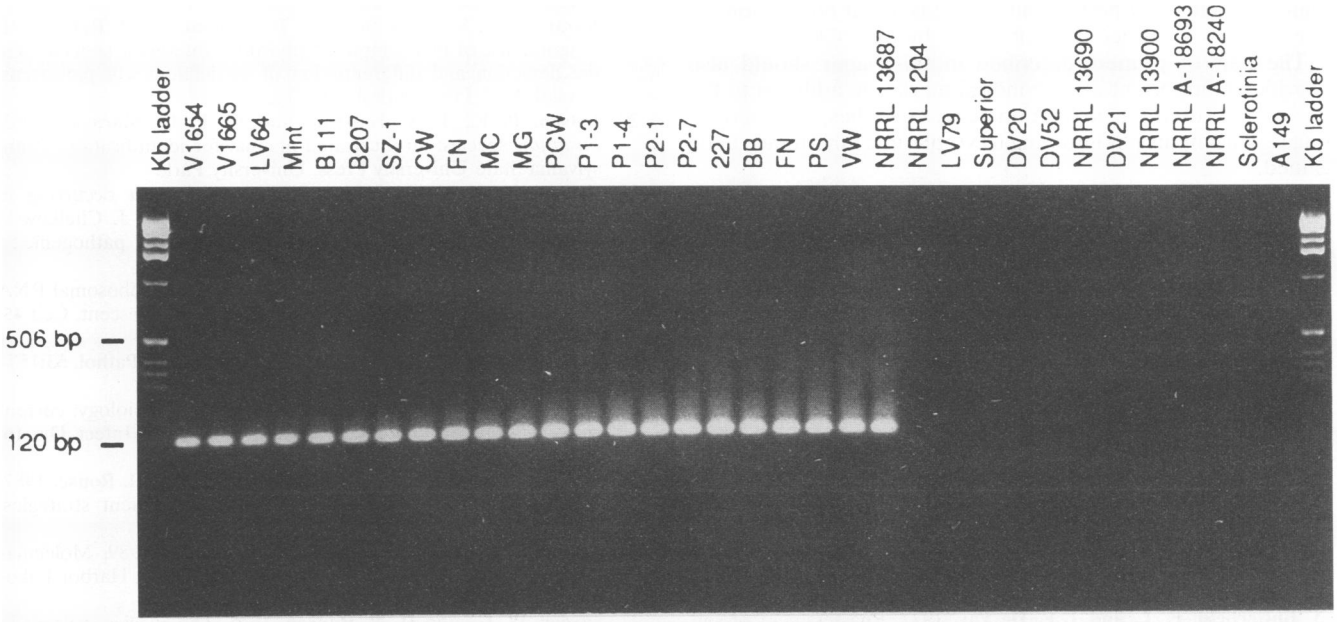

FIG. 7. Differentiation of *V. dahliae* from other fungal isolates. The negative control (TE buffer was added instead of template DNA) did not exhibit any amplification (data not shown).

If an extensively tested species-specific primer or probe were available, any questionable colonies could be isolated and treated to extract their DNAs for PCR or PCR and Southern hybridization. This procedure would result in prompt identification with a high level confidence. Our data could also be used to design a probe to differentiate *V. albo-atrum* from other fungi and thus help identify this important plant pathogen properly and promptly. While *V. tricorpus* is not as pathologically important as *V. dahliae* or *V. albo-atrum*, it is the most difficult species to distinguish from *V. dahliae* because it also produces microsclerotia (10) and thus is the most similar species morphologically. *V. tricorpus* and *V. dahliae* could be easily distinguished with primers VMSP1 and VMSP2. The results of hybridization with the 30-base probe (Fig. 5 and 6) indicated that there are more differences in the region examined between *V. tricorpus* and *V. dahliae* or *V. albo-atrum* than between *V. dahliae* and *V. albo-atrum*.

Differentiation of ascomycetes. Primers NMS1 and NMS2 may allow access to the mitochondrial small rRNA gene regions in ascomycetes and thus may be useful as specific probes at the generic or species level for any ascomycete. We will test the potential of this region in this regard. For organisms that are not well characterized molecularly, the PCR and procedures involving various rRNA genes are the most widely used approaches for differentiating closely related organisms and for locating probes that have different specificities (2). Although there are numerous pairs of primers that allow access to these regions in fungi (30), it is often difficult to obtain specific probes at the genus or species level. Usually the nuclear internally transcribed spacer regions and regions within the coding portions of the genes themselves are not divergent enough to differentiate organisms at a very specific level (2). While the nuclear nontranscribed spacer regions are believed to be very divergent (2), there are no primers that are conserved enough so that we can gain access to these regions (30). On the other hand, little is known about the potential of the mitochondrial genes for this purpose. In contrast, our newly designed primers, NMS1 and NMS2, are themselves conserved. The region between their sites is relatively divergent and may allow differentiation at the species level; the region amplified by NMS1 and NMS2 is about 600 bp long, a size which is amplified very efficiently by the PCR.

The pair of primers described in this paper should also provide an important mitochondrial marker in addition to the nuclear loci used for population genetics studies, thus permitting significant insight into fungal cytoplasmic inheritance to be gained.

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