Sequence Analysis of the ITS Regions of rDNA in *Monosporascus* spp. to Evaluate Its Potential for PCR-Mediated Detection

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This research was supported in part by a grant from the South Texas Melon Committee.

We thank M. Stanghellini, University of Arizona, M. Lobo, Centro de Semillas y Plantas de Vivero, Valencia, Spain, and T. Watanabe, Chiba Horticultural Experiment Station, Japan, for providing the isolates of *M. cannonballus,* and the late A. Alfaro-Garcia, Universidad Politecnica, Valencia, Spain, for supplying the DNA from *Acremonium* sp. We also thank V. Valadez, D. Lofland, J. Newsum, and M. Vega for technical support.

Accepted for publication 1 March 1995.

ABSTRACT

Lovic, B. R., Martyn, R. D., and Miller, M. E. 1995. Sequence analysis of the ITS regions of rDNA in *Monosporascus* spp. to evaluate its potential for PCR-mediated detection. Phytopathology 85:655-661.

Monosporascus cannonballus and *M. eutypoides* are recently described soilborne ascomycetes that cause root rot/vine decline of cucurbits. The internal transcribed spacer (ITS) regions 1 and 2 of the ribosomal DNA (rDNA) from the representative isolates of the two species were amplified by polymerase chain reaction (PCR) using conserved ITS primers from conserved regions of the 18S, 5.8S, and 28S ribosomal genes. The size of the entire spacer region, including the 5.8S gene, was estimated to be 610 bp based on gel electrophoresis of the PCR-amplified product. The entire region was cloned into a pUC18 vector, sequenced, and the DNA sequence aligned with published sequences from other fungi to identify gene-spacer junctions. Comparison to the published sequences of other fungi revealed up to 90% homology at the 3′ end of the 18S gene, up to 79% homology with the 5.8S gene and up to 74% homology at the 5′ end of the 28S gene. The DNA sequences (ITS 1 and ITS 2) contained within these regions did not show homology with any published DNA sequences. The ITS regions from 12 isolates of *Monosporascus* spp. representing all geographic regions of their reported occurrence were PCR-amplified and digested singularly with nine restriction enzymes but no length polymorphisms could be detected. The sequences of the ITS 1 and ITS 2 were used to construct five PCR primers, each of which was subsequently shown to amplify identical, predicted-size, fragments from the DNA of all 12 *Monosporascus* isolates tested. PCR primers from the ITS regions of *Monosporascus* spp. failed to amplify predicted-size fragments from the DNA of any other soilborne fungi tested including representative species of the genera considered taxonomically (e.g., *Anixiella*) and ecologically (e.g., *Fusarium*., *Stagonospora*, *Macrophomina*, etc.) most closely related to *Monosporascus* spp. These primers were also tested on the DNA extracted from the roots of *Monosporascus*-infected muskmelon and were shown to consistently amplify the predicted-size fragments whose identity was further confirmed by Southern hybridization to the digoxigenin-labeled portions of the ITS regions. In addition to establishing that the DNA sequence of the ITS region is conserved within the genus *Monosporascus* this study also illustrates a convenient approach to developing "molecular tools" for detection of plant pathogenic fungi without necessarily having any detailed knowledge of their genome organization.

Additional keyword: PCR diagnostics.

Monosporascus cannonballus Pollack & Uecker is a recently described ascomycete (32,36) and the causal agent of Monosporascus root rot/vine decline of muskmelon (*Cucumis melo* L.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) in the Lower Rio Grande Valley of South Texas (25,26,27). In addition to Texas, this organism has been reported from Arizona and California (M. Stanghellini and T. Gordon, *personal communication*) in the United States, and also has been reported to cause a similar disease in Japan (42,44) and Spain (17). *Monosporascus eutypoides* (Petrak) Von Arx is the only other species currently in this genus (37) and it has been associated with the symptoms of sudden death and vine decline of melons in Israel (33). Another putative species classified in this genus is *Monosporascus monosporus* (Malloch & Cain Comb. nov.) Hawksworth & Ciccarone (8,21); however, it is only known from a holotype description and there is no known viable culture (21).

M. cannonballus and *M. eutypoides* are found in almost identical hot climatic habitats and cause very similar, if not identical symptoms on their hosts: reduced growth during the season, progressive defoliation after fruit set, and partial or complete collapse of the affected plants toward the end of the season (13,17, 25,33). Inspection of the roots of declined plants reveals root rot, necrosis, and cortex sloughing. Perithecia form late in the season, are embedded in the cortical tissue, and typically contain 150 to 200 asci, each with one to two large, thick-walled, spherical ascospores (25,32,36,37). No conidial stage has been reported for either species.

As described (36,37) the two species are similar, if not identical, in all morphological characters except in the number and germinability of mature spores within the ascus; *M. cannonballus* consistently has one spore per ascus (36) while *M. eutypoides* is reported to have predominantly two-spored asci (37). Also, *M. cannonballus* ascospores could not be induced to germinate under standard laboratory conditions (25,32) while ascospores of *M. eutypoides* germinate readily (33,37). However, as many as 8% of the ascospores produced by Japanese isolates of *M. cannonballus* were reported to germinate readily (41) and Martyn et al. (22) reported a 0.5% germination rate of the ascospores produced by Texas isolates following some laboratory manipulation.

Molecular systematics is increasingly being used to provide solutions in situations in which classical taxonomic characters fail to provide convincing evidence for a consensus classification scheme (2,3,28). Ribosomal DNA (rDNA) has proven to be especially valuable in this effort (4,5,7,15,16,43,45) since different coding and noncoding regions of rDNA evolve at different rates

and provide optimal levels of sequence variation for differentiating between a range of taxonomic categories, from kingdoms to clones (3,11). The internal transcribed spacer (ITS) regions of rDNA have been shown to be generally conserved at the species level but variable in higher taxa (3) making them particularly useful for species differentiation. However, the ITS DNA sequence was shown to be highly variable in several fungi, allowing for subspecies differentiation and phylogenetic analysis (30,43).

The primary reason for analyzing the DNA sequence of the ITS regions in *Monosporascus* spp. was to identify a unique DNA sequence for construction of primers suitable for polymerase chain reaction (PCR)-based identification and detection. Soilborne plant pathogenic fungi are notoriously difficult to detect and identify but certain characteristics of *Monosporascus* spp. make this problem even more pronounced. For example, ascospores are the only spore stage described and they do not germinate readily under standard laboratory conditions. The fungus can be isolated from the infected roots and tentatively identified based on the characteristics of its mycelium; however, it still requires an average of 3 to 4 weeks in culture for perithecia to form (25,32). In addition, a large proportion of field isolates are variable in their morphology and produce perithecia inconsistently in culture (18,19). The sensitivity of PCR-based detection (1,34) and its applicability for detection in minimal amount of tissue (14,45) and complex environments such as roots (10,12,29) or soil (38,39), make it an ideal choice for overcoming current constraints on identification and detection of *Monosporascus* spp.

In this report we describe PCR amplification and restriction enzyme analysis of the ITS regions from a collection of *M. cannonballus* isolates representing all geographic areas of their reported occurrence. We further establish that the DNA sequence of the ITS regions is conserved within the genus *Monosporascus* and describe a series of internal primers. We show these primers to be specific to *Monosporascus* spp. by testing them against the DNA from a number of fungi believed to be taxonomically and ecologically most closely related to *Monosporascus* spp. and illustrate the usefulness of these primers for PCR-based identification and detection of *Monosporascus* spp. in muskmelon roots. A preliminary report of this work has been published (20).

MATERIALS AND METHODS

Fungal isolates and culture maintenance. Twelve isolates of *Monosporascus* spp. identified as either *M. cannonballus* or *M. eutypoides* used in this study are listed in Table 1. Active cultures of each isolate were grown on V8-juice agar and stored in sand/wheat hull medium (20:1, vol/vol) for long-term preservation (25). The isolates of *Fusarium solani* (Mart.) Sacc. emend W. C. Snyder & H. N. Hans., *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *melonis* (Leach & Currence) W. C. Snyder & H. N. Hans., *Rhizoctonia solani* Kühn, *Macrophomina phaseolina* (Tassi) Goidanich, *Pythium ultimum* Trow, and *Stagonospora* sp. were collected from muskmelon roots in South Texas and identified in our laboratory. The isolate of *Anixiella endodonta* Malloch and Cain (ATCC #18985) was obtained from the American Type Culture Collection (Rockville, Md.).

TABLE 1. Isolate designation, original host, geographic origin and source of *Monosporascus* spp. used in this study

Isolate	Host	Origin	Source
AZ90-33	Cantaloupe	Arizona (1974)	ATCC 26931
CA91-16	Cantaloupe	California (1990)	M. Stanghellini
ESP92-1	Cantaloupe	Spain (1989)	M. Lobo
ESP92-2[a]	Cantaloupe	Spain (1989)	IMI 345145
ESP93-3[a]	Cantaloupe	Spain (1990)	A. Alfaro
ESP93-5[a]	Cantaloupe	Spain (1990)	A. Alfaro
JPN91-20	Cantaloupe	Japan (1989)	T. Watanabe
JPN91-21	Cantaloupe	Japan (1989)	T. Watanabe
TX90-25	Cantaloupe	Texas (1989)	J. Mertely
TX91-18	Watermelon	Texas (1990)	J. Mertely
TX92-4	Honey Dew Melon	Texas (1992)	B. Lovic
TX92-10	Cantaloupe	Texas (1992)	B. Lovic

[a] These isolates were classified as *Monosporascus eutypoides* at the International Mycological Institute, Surrey, U.K.

Cultural conditions and DNA isolation. For DNA extractions, mycelium was grown in modified Fusarium liquid culture medium (6) using sorbitol as a carbon source, 1 g per liter of yeast extract, and adjusting the pH to 7.0. One-hundred-milliliter or 150-ml volumes were seeded with a single 9-mm agar plug from the colony edge of a 3-day-old culture and incubated as still cultures at room temperature for 10 days. Mycelium was harvested by vacuum filtration through Miracloth (Calbiochem-Novabiochem Corporation, La Jolla, Calif.), washed with sterile distilled water, and immediately stored at –80°C for 1 to 14 days before lyophilizing. Dried mycelium was crushed and the DNA extracted by treating with CTAB extraction buffer (700 mM NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid, disodium salt [EDTA],10% hexadecyltrimethylammonium bromide [CTAB]) followed by phenol/chloroform purification and precipitation in isopropanol and ethanol (24).

PCR amplification. PCR primers (nos. 1, 2, 3, and 4) used to amplify the ITS region were described previously (45) and their location within the rDNA repeat unit is indicated in Figure 1A. These are referred to as "conserved ITS primers" since they are from the conserved areas of the 18S and 28S genes and have been shown to amplify the ITS regions and 5.8S gene from a number of distantly related organisms. PCR amplifications were performed in 30- or 100-ml volumes containing 1 mM each of the primers; 500 mM of each of the four deoxyribonucleotides; 2.5 mM $MgCl_2$; 0.6 and 2.0 (for 30- and 100-µl reactions, respectively) units of *Taq* polymerase in 1X PCR buffer (Promega, Madison, Wis.). Temperature parameters were 94°C for DNA denaturation, 5 min for the first cycle and 1 min for the remaining

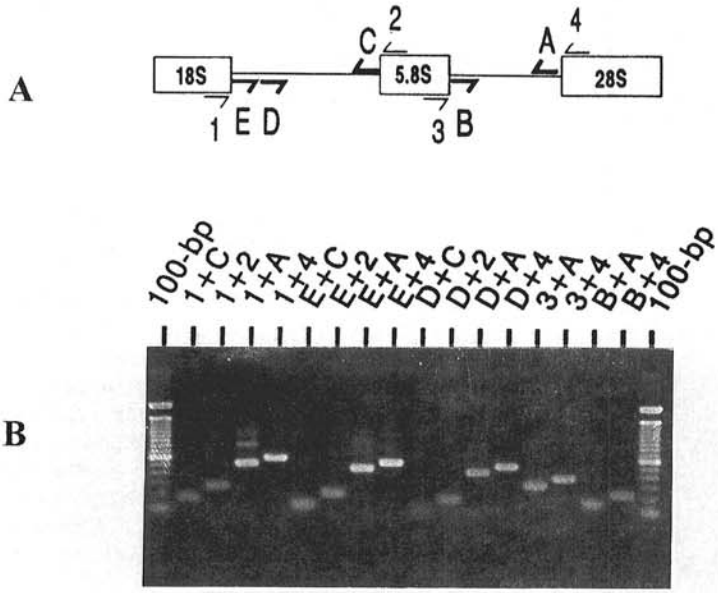

Fig. 1. Location of the primers along the ribosomal DNA repeat unit and polymerase chain reaction (PCR) amplification products using all primer combinations. **A,** Schematic representation of ribosomal DNA repeat unit. Location of conserved primers is shown by numbers (1 through 4) while the primers constructed based on the DNA sequence of the internal transcribed spacer regions (Table 2) are represented by letters (A through E). **B,** Agarose gel electrophoresis of PCR products following 25 amplification cycles using different primer combinations and the DNA from *Monosporascus cannonballus.*

cycles, 45°C for 1 min for primer annealing, and 72°C for 1 min for primer extension. The total number of cycles was 25 plus a final extension of 7 min. The reactions were run using the Perkin-Elmer Cetus 480 thermal cycler (Perkin-Elmer, Norwalk, Conn.). The amplified products were analyzed by electrophoresing 5-µl aliquots through a 1.2% agarose gel, staining with ethidium bromide, and visualizing under ultraviolet (UV) light (35).

Restriction fragment length polymorphism analysis of the ITS regions. PCR-amplified products using conserved ITS primer pair 1 and 4 were used directly for restriction enzyme digestion with nine restriction enzymes (GIBCO, BRL, Gaithersburg, Md.) according to manufacturer's instructions. The enzyme-digested DNA was electrophoresed through a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Cloning and sequencing of the ITS region. The ITS regions from two isolates (*M. cannonballus* ATCC strain #26931 and *M. eutypoides* IMI strain #345135) were cloned and one clone of each isolate was sequenced. The product of the amplification reaction was used directly for ligation into a plasmid vector (pUC18) using Sure Clone Ligation Kit (Pharmacia, Piscataway, N.J.) and transformed into competent *Escherichia coli* cells (strain DH5α) according to the manufacturer's instructions. DNA sequence was determined in Gene Technologies Laboratory (Texas A&M University, College Station) using dideoxy chain-terminating Sequenase 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio) and universal sequencing primers for the vector. The DNA sequence was further confirmed by sequencing the PCR product directly using primers 1 and 4 (Fig. 1). The identity of the amplified fragment was determined by sequence alignment with published sequences from the rDNA regions amplified by the same primers from *Fusarium sambucinum* (30). The 5′ and 3′ regions of each ITS shared greatest percent homology with the same rDNA regions in *F. sambucinum* (DNA Sequence Database, National Institute of Health [NIH], Bethesda, Md.).

Primer synthesis and testing. Five primer sequences (Table 2) within the ITS regions 1 and 2 were selected based on their positions along the amplified fragment and an average G-C content ranging from 55 to 60%. These are referred to as primers A, B, C, D, and E (Fig. 1A) and were synthesized in the Oligonucleotide Synthesis Lab at the Biology Department, Texas A&M University. The primers were tested for the amplification of the predicted-size fragments and for specificity to *Monosporascus* spp. using the PCR amplification conditions as described above.

Southern analysis. Amplification products were separated by electrophoresis in a 1.2% agarose gel and transferred onto a positively charged nylon membrane according to manufacturer's instructions (Boehringer Mannheim, Indianapolis, Ind.). Hybridization probes were constructed by digoxygenin labeling of the PCR-amplified portions of the ITS region(s) and the 5.8S gene using Genius labeling kit (Boehringer Mannheim). Hybridization probes were constructed from the amplification products using primers A+B, A+E, and C+E (Fig. 1A). The hybridizations were performed according to the manufacturer's instructions (Boehringer Mannheim).

Tests for primer specificity. Primer specificity was evaluated against 13 species in 10 genera. The DNA from *Fusarium oxysporum* and *F. solani*, *R. solani*, *M. phaseolina*, *P. ultimum*, *Stagonospora* sp., and *A. endodonta* was extracted using the same protocol as described for *M. cannonballus*. The DNA from the isolates of *Sclerotium rolfsii* Sacc., *Sclerotinia sclerotiorum* (Lib.) de Bary, *Aspergillus parasiticus* Speare, and *A. flavus* Link were kindly provided by Nancy Keller (Department of Plant Pathology and Microbiology, Texas A&M University). DNA from an isolate of *Acremonium* sp. was kindly provided by Agustin Alfaro-Garcia (Departamento de Patologia Vegetal, Universidad Politecnica, Va-

TABLE 2. Sequence and location of the polymerase chain reaction primers within the internal transcribed spacer region of the rDNA in *Monosporascus* spp.

Primer ID	Sequence	Location
A	CGCTGGCTTCTGGCCACTAAACC	501-523
B	GCTTGGTGTTGGGAGCTTATCGC	391-413
C	CCGGTGGACCATGTAAACTCTT	151-172
D	GAGTAGCCTACCCGGTAGCTAC	107-128
E	CTTACCTATGTTGCCTCGGCG	60-80

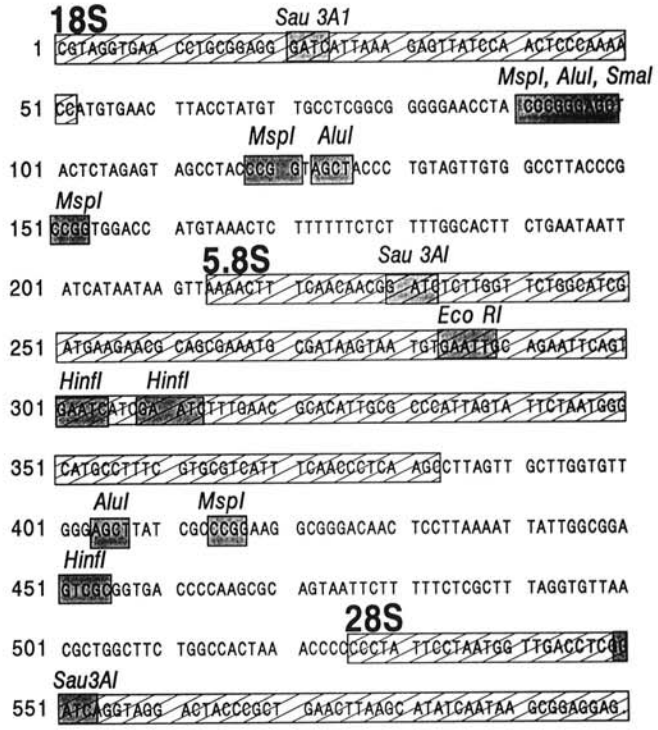

Fig. 2. DNA sequence of the internal transcribed spacer region of *Monosporascus cannonballus*. Portions of the sequence sharing homology with the 18S, 5.8S, and 28S portions of the ribosomal DNA repeat unit of other fungi are boxed in open frames. The sequences of the restriction sites for the nine enzymes used for restriction fragment length polymorphism analysis is represented by gray boxes.

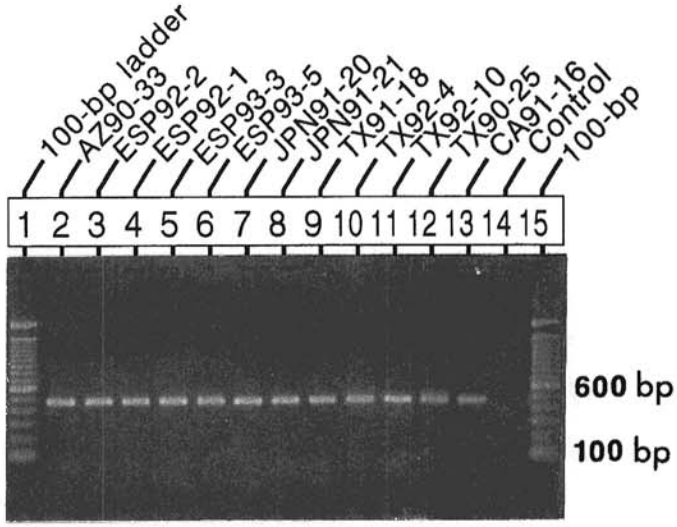

Fig. 3. Agarose gel electrophoresis of the polymerase chain reaction–amplified products from the DNA of geographically separated isolates of *Monosporascus cannonballus* using primers A and E. Expected-size (480-bp) fragment is observed in all lanes. Origin and source of isolates is described in Table 1.

lencia, Spain). The amplification conditions for the PCR were the same as described for *M. cannonballus*.

PCR amplification from muskmelon roots infected by *M. cannonballus*. Muskmelon plants (cultivar Magnum 45) were grown for 60 days in 3-liter (6-inch) black plastic pots in a mixture of pasteurized sand/perlite/peat (5:1:1) infested with *M. cannonballus* mycelium as described previously (27). At the end of the experiment roots were harvested and evaluated for occurrence of perithecia and extent of root rot. Root systems that had no visible perithecia and only minor necrosis and root rot were selected and used for DNA extraction and PCR amplification. Roots were washed thoroughly in tap water, rinsed with double-distilled water, blotted dry, cut into 1-cm segments and stored at –80°C until used. DNA was extracted from 2-g root samples following homogenization in liquid nitrogen and a standard CTAB protocol (24). The DNA concentrations were determined in a fluorimeter (Hoefer Scientific, San Francisco, Calif.), adjusted to 50 mg/ml with sterile double-distilled water and 1-ml aliquots used for PCR amplifications. Parameters of the PCR were as described above, except that the annealing temperature was increased to 50°C and the total number of cycles was 45.

RESULTS

Characteristics of the ITS DNA sequence of *Monosporascus* spp. Total length of the amplification product using conserved ITS primers 1 and 4, including the ITS 1, ITS 2, and the 5.8S gene, was determined to be 610 bp based on agarose gel electrophoresis (Fig. 1B, lane 5) and 599 bp based on DNA sequencing (Fig. 2). The size of the PCR fragment and its sequence in representative isolates of *M. cannonballus* and *M. eutypoides* were identical. Sequence alignment with published sequences from other fungi revealed up to 90% homology at the 3′ end of the 18S gene (bases 1 to 52), up to 79% homology with the 5.8S gene (bases 214 to 383) and up to 74% homology with the 5′ end of the 28S gene (bases 526 to 599). The DNA sequence of the ITS 1 (bases 53 to 213) and ITS 2 (bases 384 to 525) did not show homology with any reported DNA sequences (NIH database, Bethesda, Md.).

Based on the 599-bp sequence (Fig. 2), six restriction enzymes (*Alu*I, *Eco*RI, *Hin*fI, *Msp*I, *Sau*3AI, and *Sma*I) with restriction site(s) within the ITS-5.8S sequence and three enzymes (*Hae*II, *Rsa*I, *Sal*I) without restriction sites within this sequence were selected. The restriction digests were performed on PCR-amplified products (using primers 1 and 4) from the DNA of the 12 isolates of *Monosporascus* spp. from geographically separated areas. None of the nine enzymes revealed any polymorphisms in the ITS fragment suggesting no differences among the 12 isolates tested (data not shown).

Construction and testing of the *Monosporascus*-specific primers. Five 21- or 23-base-pair sequences with a GC content of 55 to 60% were selected from the DNA sequence of the ITS regions 1 and 2 of *M. cannonballus* isolate AZ90-33 (Table 2). The position of these sequences and the orientation of the primers are shown in Fig. 1A. These primers along with the conserved ITS primers 1, 2, 3, and 4 (45) from the conserved regions flanking the ITS part of the ribosomal repeat unit amplified the products of predicted sizes when used in all possible combinations (Fig. 1B). The PCR products were amplified from the DNA of a collection of geographically separated isolates. The same uniformity in predicted size observed for PCR amplification products using primer combination A+E (Fig. 3) was observed with primer combinations A+D and C+E (data not shown).

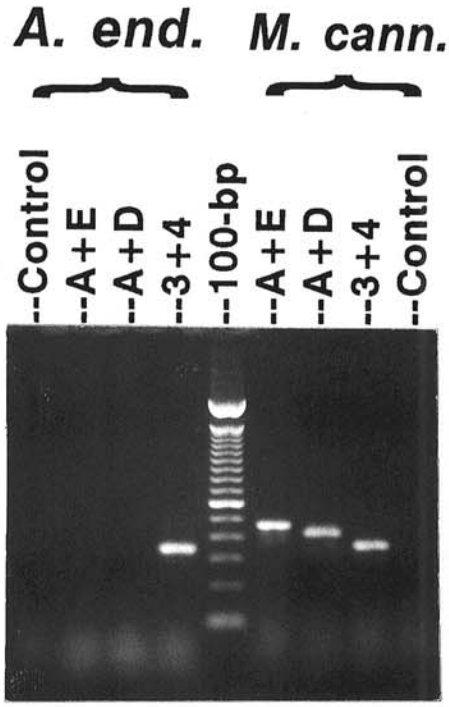

Fig. 4. Agarose gel electrophoresis of polymerase chain reaction amplification products using conserved internal transcribed spacer and *Monosporascus*-specific primers (Table 2) and the DNA from *M. cannonballus* and *A. endodonta.*

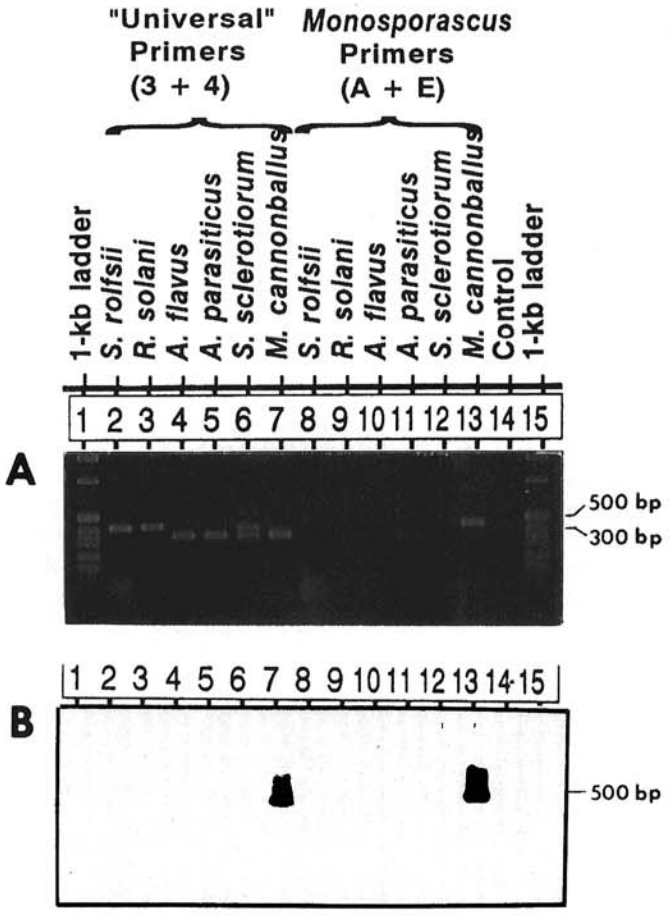

Fig. 5. Agarose gel electrophoresis and Southern hybridization of the polymerase chain reaction (PCR) amplification products using conserved ("universal") internal transcribed spacer primers 3 and 4 and *Monosporascus* primers A and E with DNA from *M. cannonballus* and several other soilborne fungi. **A,** Agarose gel electrophoresis of 10 µl of the amplification products following 24 cycles of PCR. **B,** Southern hybridization using digoxigenin-labeled product of PCR amplification using primers A and B (Fig. 1).

Tests for primer specificity. Primers A, B, C, D, and E did not amplify the DNA of *A. endodonta*, a taxonomically related species (Fig. 4), or the DNA from a number of other soilborne fungi (Fig. 5). In the case of some species (e.g. *Aspergillus parasiticus*, Fig. 5), products of lower fluorescence intensity could be observed; however, none of those corresponded to the expected fragment sizes and no DNA sequence homology was detected by hybridization with the *Monosporascus* ITS probe (Fig. 6A). Other fungi tested included *Fusarium solani*, *F. oxysporum*, *Acremonium* sp., *Macrophomina phaseolina*, and *Stagonospora* sp., and no products of corresponding size or homologous DNA sequence were amplified (data not shown). In contrast, the conserved ITS primer pair 3+4, included as a positive control, amplified the DNA from each of the species, indicating that the amplification conditions were optimal (Figs. 5 and 6).

Detection of the pathogen in muskmelon roots. In preliminary tests, primer pair A+D yielded the most consistent and most efficient amplifications (data not shown) and was used in all later experiments. The predicted-size fragment amplified by primer pair A+D (430 bp) was consistently amplified from infected roots and could be observed in ethidium bromide–stained gels as illustrated in Figure 6. In each case the identity of the fragment was confirmed by hybridization with the digoxigenin-labeled portion of the *Monosporascus* ITS region (Fig. 6). A digoxigenin-labeled 100-bp ladder was included in hybridization solution to allow for size comparison on autoradiographies.

DISCUSSION

Our data suggest that the DNA sequence of the ITS regions of rDNA repeat unit of *Monosporascus* spp. is homogeneous within this genus yet different from that of other taxonomically and ecologically related fungi. Lack of divergence within the ITS is suggested by three lines of evidence. First, restriction enzyme analysis using six four-base cutting and three six-base cutting enzymes failed to detect any restriction fragment length polymorphisms among a collection of geographically separated isolates of *Monosporascus* spp. Secondly, five 21-23-bp primers constructed based on the ITS sequence amplified products of predicted sizes from all *Monosporascus* isolates tested. Lastly, the DNA sequence of the representative isolates of *M. cannonballus* and *M. eutypoides* from Texas and Spain, respectively, was identical over a 599-bp stretch from the 3′ end of the 18S gene to the 5′ end of the 28S gene. Thus, the DNA sequence of the ITS region has little value as a phylogenetic tool in this genus, but is potentially useful for PCR diagnostics.

Complete identity of the ITS DNA sequences in the representative isolates of *M. cannonballus* and *M. eutypoides* is inconsistent with the current view of the variability within the ITS region in fungi (3,16). This region is generally viewed as conserved within a species yet variable among species of the same genus (3); however, a recent study of sequence variation among the genetically fertile strains of *Fusarium sambucinum* revealed extensive sequence divergence even within the same biological species (30).

We have previously suggested that *M. cannonballus* and *M. eutypoides* may be synonymous (23). The two species are differentiated based on the number of spores per ascus (*M eutypoides* has predominantly two while *M. cannonballus* has predominantly one) and germinability of the spores (*M. eutypoides* ascospores germinate readily unlike those of *M. cannonballus*) (36,37). Both of those arguments have been challenged. In our laboratory we have never observed germination of ascospores of *M. eutypoides* (strain IMI 345135) over a 2-year period. Additionally, we demonstrated that a very small proportion of *M. cannonballus* ascospores germinated following some laboratory manipulation (22). Also, the IMI isolate of *M. eutypoides* was observed to consistently produce asci containing a single spore of a shape and size indistinguishable from that of the isolates of *M. cannonballus* (R. D. Martyn, *unpublished data*). Additionally, this isolate was originally described as having only one spore per ascus yet identified as *M. eutypoides* (P. F. Cannon, International Mycological Institute, Surrey, U.K., *personal communication*). Finally, we have also observed a rare occurrence of two- and three-spored asci in at least one isolate of *M. cannonballus* (23). In light of these data and observations, the fact that the DNA sequence of the ITS region of the ribosomal DNA in both *Monosporascus* species is identical may not be in contrast to the general understanding of the variability within the ITS region in fungi. Rather, this fact supports the view that *M. cannonballus* and *M. eutypoides* are synonymous.

The conserved nature of the ITS sequence in *Monosporascus* spp. and the fact that fragments of predicted size can be amplified from the asymptomatic roots grown in *Monosporascus*-infested soil suggest the potential of these PCR primers in pathogen detection and diagnostics. PCR-based detection is becoming an increasingly popular approach to plant disease diagnostics and it is a particularly useful approach in cases of diseases of complex etiology (9) such as Monosporascus root rot/vine decline.

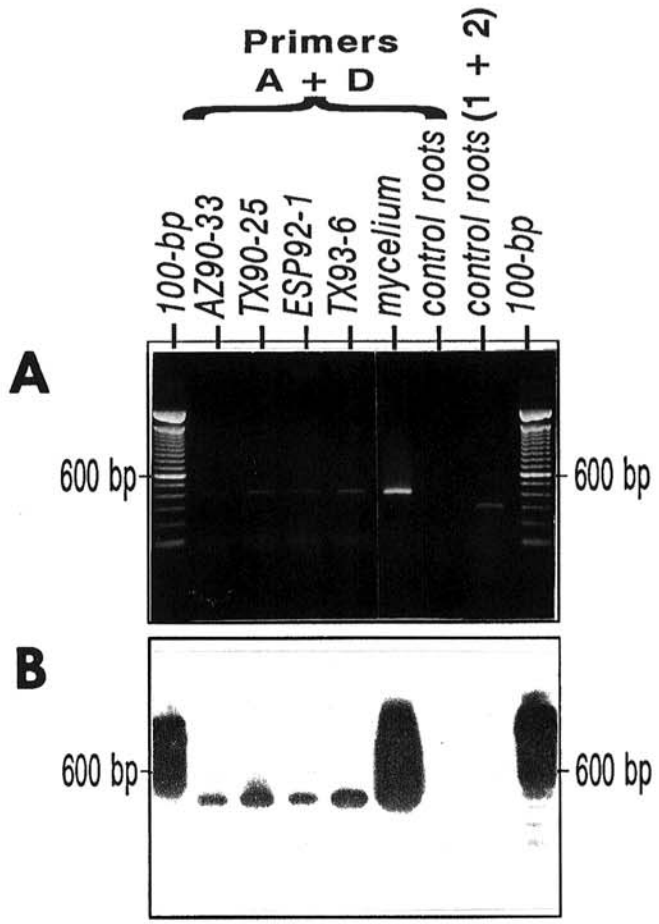

Fig. 6. Agarose gel electrophoresis and Southern hybridization of the polymerase chain reaction (PCR) amplification products using primers A and D and the DNA from roots infected with four different isolates of *Monosporascus cannonballus*. **A,** *Agarose* gel electrophoresis of 10-ml aliquots of PCR amplification products using primers A and D and the DNA from four muskmelon roots, each infected with a different isolate of *M. cannonballus* (TX90-25, AZ90-33, TX90-25, TX93-6). DNA extracted from mycelium of the standard isolate and from control (noninfected) roots served as a positive and negative control, respectively. Amplification product from the DNA from noninfected control using "conserved" primers 1 and 2 is also shown. **B,** Southern hybridization (probe A + B, Fig. 1) confirms the identity of the PCR products from infected roots as *M. cannonballus*. Digoxigenin-labeled 100-bp ladder was included into hybridization solution.

The series of primers and probes constructed in this study should provide an array of tools for developing a sensitive and convenient detection method for this organism in root tissue and soil. Forty-five cycles of PCR amplification using *Monosporascus*-specific primers A+D produced a sufficient amount of the predicted-size fragment to be visualized on ethidium bromide–stained gels. However, the fragments were much more easily identified after Southern transfer and hybridization with the digoxigenin-labeled portions of the ITS region. Employment of the additional hybridization step increased the detection limit in similar assays for plant virus detection at least 10-fold (31). The five primers could also allow for performing two sets of amplifications using nested primers in the second run (1,9). The first round of PCR amplification could be performed using primers A and E (Fig. 1A) and the product could be used in the second round of amplifications using primers C and D. Besides increasing the specificity and sensitivity of the test, the use of nested primers dilutes the DNA polymerase inhibitors that may be present in plant or soil samples (9,10).

This study illustrates a convenient approach to developing "molecular" tools for detection of plant pathogenic fungi without necessarily having any prior knowledge of their genome organization. *Monosporascus*-specific primers suitable for detection were identified by sequencing a relatively short stretch of DNA, followed by PCR and restriction enzyme analysis of the PCR-amplified products from a collection of geographically separated isolates. Besides developing the ITS primers and probes into a detection method for *Monosporascus* spp., this study illustrates a convenient approach to developing species-specific primers and probes for other fungi believed involved in the melon vine decline disease complex (e.g. *Fusarium solani*, *F. oxysporum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Stagonospora* spp., etc.).

LITERATURE CITED

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