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PCR-Based Strategy To Detect and Identify Species of *Phaeoacremonium* Causing Grapevine Diseases▽

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Species of *Phaeoacremonium* (especially *Phaeoacremonium aleophilum*) are associated with two severe diseases in grapevines, Petri disease in young plants and Esca disease in adult plants. *Phaeoacremonium* species grow slowly on culture medium, and it is difficult to identify these species on the basis of morphological characteristics. Primers Pm1 and Pm2 were designed in the ribosomal DNA internal transcribed spacer (ITS) regions ITS1 and ITS2, respectively. They yielded a single amplicon of 415 bp for nine species of *Phaeoacremonium* that may occur in grapevines. A nested PCR (using general fungal primers ITS1F/ITS4 in the primary reaction) was developed to detect *Phaeoacremonium* directly in grapevine wood. Molecular detection was more sensitive than the traditional method of culturing in growth medium was. Identification of *Phaeoacremonium* species was achieved by digesting the PCR-amplified fragment with the restriction enzymes BssKI, EcoO109I, and HhaI. It was possible to distinguish these species by their restriction fragment length polymorphism patterns, except for *Phaeoacremonium viticola* and *Phaeoacremonium angustius*, which had 100% similarity in their ITS region sequences. A species-specific PCR amplification of the partial β-tubulin gene using the primer pair Pbr4_1/T1 and Pbr8/T1 was necessary to differentiate *P. angustius* from *P. viticola*, respectively. An easy and fast protocol was developed to detect and identify species of *Phaeoacremonium* in a few hours. Primers defined here can be used in a plant nursery sanitation program to produce plants free of *Phaeoacremonium* spp. Use of healthy grapevine plants in new plantations is the most effective measure to manage Petri disease.

The hyphomycete genus *Phaeoacremonium* W. Gams, Crous & M. J. Wingfield is an ecologically important taxon that includes species associated with declining disease of woody plants and infections in humans (3, 8, 18, 25). In grapevine (*Vitis vinifera* L.), the two most serious destructive declining diseases are Petri disease in young plants and Esca disease in adult vines. The main pathogens identified in Petri disease are *Phaeoacremonium* spp. (most frequently *Phaeoacremonium aleophilum*) and *Phaeomoniella chlamydospora* (formerly known as *Phaeoacremonium chlamydosporum*) which are associated with the internal wood symptoms of dark brown to black streaking in a longitudinal section of stems. The importance of Petri disease has been emphasized, since it has been suggested these fungi act as pioneer organisms in the later invasion of the wood decay fungi that cause the typical symptoms of Esca disease inside the trunk and branches (23).

The genus *Phaeoacremonium* was described in 1996 (3) and included some plant- and human-infecting isolates grouped with the name of *Phialophora parasitica*. Six new species, including the type species *Phaeoacremonium parasiticum*, were described then (3). The other *Phaeoacremonium* species were *P. aleophilum*, *P. angustius*, and *P. chlamydosporum* isolated from grapevines and *P. inflatipes* and *P. rubrigenum* isolated from humans and plants (including vines). In later studies, *P. chlamydosporum* appeared unrelated phylogenetically to other species of the genus (7), and it was renamed *Phaeomoniella chlamydospora* (4). Two new *Phaeoacremonium* species were later described: *P. mortoniae* (17) and *P. viticola* (8). Subsequent DNA phylogenetic study of the internal transcribed spacer (ITS) region, ITS1/5.8S gene/ITS2, and especially of the β-tubulin, actin, and calmodulin gene regions of the *Phaeoacremonium* species brought the description of an important number of novel species or the reassignment of former ones. Thus, the *Phaeoacremonium* genus grew to include five new species, *P. australiense*, *P. krajdenii*, *P. scolyti*, *P. subulatum*, and *P. venezuelense*, that grow on grapevines (24); *P. rubrigenum* was shown to occur only on humans (24). Recently, two other new *Phaeoacremonium* species on grapevines were defined, *P. austroafricanum* and *P. iranianum* (25). This makes a total of 13 *Phaeoacremonium* species that are reported to grow on grapevines.

Identification of species of *Phaeoacremonium* is not easy. It is done by traditional methods of isolation and culturing and subsequent description of morphological characteristics. There are some morphological identification keys (3, 8, 24), but distinguishing between the characteristics has proven to be difficult and it has resulted in some misidentifications. Moreover, *Phaeoacremonium* spp. are slow-growing fungi which usually take up to 20 days to grow on enriched medium. *Phaeoacremonium* is frequently overgrown by other microorganisms; then subculturing is required, which makes the identification process longer. Molecular tools have contributed to identify *Phaeoacremonium* species. Restriction patterns of the ITS ribosomal DNA (rDNA) and a partial fragment of the β-tubulin gene were used to distinguish *Phaeoacremonium parasiticum* from *Phaeoacremonium inflatipes* (9) and to identify some of the *Phaeoacremonium* species associated with diseased grapevines (9, 33). Species-specific primers based on ITS region of rDNA have been widely used to detect and identify *Phaeoacre-*

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TABLE 1. *Phaeoacremonium* isolates used in this study

Phaeoacremonium species	No. of isolates	GenBank accession no.[a]	Origin	Yr of isolation
P. parasiticum		U31841	CBS860.73[b,c,d]	1973
			CBS113591[e]	
	2 (1)[f]		Plant nursery in Murcia, Spain	2004
	2 (1)		Plant nursery in Cataluña, Spain	2005
	1 (1)		Cataluña, Spain	2005
P. rubrigenum		AF118139	CBS498.94[c]	1994
P. inflatipes		AF197990	CBS391.71[c,d]	1966
			CBS166.75[e]	1974
P. mortoniae		AF295328	CBS211.97[e]	
			CBS101585[c,d]	1998
P. viticola		AF118137	CBS113065[e]	2001
			CBS101738[c,d]	1993
	1 (1)		Cataluña, Spain	2005
P. angustius		AF197974[g]		
			CBS114992[c,d]	1992
			CBS114991[e]	
P. krajdenii			CBS109479[d]	2001
P. venezuelense			CBS651.85[d]	1974
P. scolyti			CBS113593[d]	
			CBS113597[e]	1999
P. aleophilum		AF017651	CBS246.91[c,d]	1996
			CBS110753[e]	1998
	1		Toscana, Italy	
	16 (1)		Ciudad Real, Spain	2003
	2 (2)		Ciudad Real, Spain	2004
	4		Cataluña, Spain	2005
	2		Plant nursery in Portugal	2004
	5 (1)		Plant nursery in Valencia, Spain	2004
	1		Plant nursery in Murcia, Spain	2004
	2		Plant nursery in Cataluña, Spain	2005

[a] GenBank accession numbers of the ITS sequences used to design the primers and the discriminant restriction enzyme digestion patterns.
[b] CBS isolates are from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.
[c] Ex-type strains used to optimize PCR conditions.
[d] Ex-type strains used to test PCR-RFLPs.
[e] CBS isolates used to validate the identification by PCR-RFLP.
[f] The numbers in parentheses are the numbers of field isolates identified by ITS sequences (and β-tubulin for *P. viticola*) used to validate the identification by PCR-RFLP.
[g] ITS sequence of CBS isolate 249.95. This was the original holotype of *P. angustius*, which was replaced with CBS114992 when lethally contaminated.

monium aleophilum and *Phaeomoniella chlamydospora* from a worldwide range of sources (5, 16, 28, 30, 33, 36). A set of species-specific primers targeting the β-tubulin and actin gene have been designed for each of the 22 species in the *Phaeoacremonium* taxon (25). These primers can be combined in a multiplex PCR to identify simultaneously at most two species of *Phaeoacremonium*.

Management of young grapevine declining disease or Petri disease relies on the use of pathogen-free plants for new plantings (32). Infection may take place in plant nurseries during the propagation process and storage (29, 37). It may also happen because of the use of infected mother plants (10, 12). Rootstock material used for propagation has been reported to harbor trunk disease pathogens and especially *Phaeoacremonium* species (1, 11, 13, 15, 30). *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. have been detected in both symptomatic and asymptomatic cuttings (2).

The aims of this work were, first, to design *Phaeoacremonium*-specific primers for the detection of any species of *Phaeoacremonium* infecting grapevine and, second, to develop an accurate restriction pattern of the resulting amplicon for the identification of the *Phaeoacremonium* species.

MATERIALS AND METHODS

Isolates of *Phaeoacremonium*. A total number of 56 *Phaeoacremonium* isolates were used in this study (Table 1). Thirty-nine isolates were isolated in 2003, 2004, and 2005 from diseased grapevines collected in different locations or rootstocks from plant nurseries in Spain. Seven ex-type cultures obtained from the Centraalbureau voor Schimmelcultures (CBS) (Table 1) were used to optimize conditions for amplifying a *Phaeoacremonium*-specific DNA fragment. Nine ex-type strains (including the first seven species and three new defined species and discarding *P. rubrigenum* because it was not longer reported to occur on grapevines) (Table 1) were used for verifying the restriction digestion pattern of the amplicon. Other CBS cultures and field isolates were used to evaluate the method presented here (Table 1). Each isolate was plated on 2% malt extract agar (MEA) (Conda Laboratories, Torrejón de Ardoz, Madrid, Spain) and incubated at 25°C in the dark in order to obtain mycelium for DNA extraction. All isolates were stored at 4°C in sterile water.

Other fungal isolates. Other fungal isolates collected from grapevines (Table 2) were tested to verify that PCR primers were specific for the amplification of *Phaeoacremonium* DNA. They were obtained from the same surveys as *Phaeoacremonium* isolates were. These isolates were previously identified (1) on the basis of their morphology (3, 4, 6, 8, 27, 35) or their identification by a BLAST search in the GenBank database using the ITS region.

DNA extraction. Fungal DNA was extracted from either fresh mycelium ground with liquid N_2 or freeze-dried mycelium, using the commercially available DNeasy plant mini kit (QIAGEN, Hilden, Germany). DNA samples were kept at −20°C until they were used for PCR amplifications.

Primer design. ITS sequences of seven holotype species of *Phaeoacremonium* were used to design a genus-specific primer pair (Table 1). ITS sequences were

TABLE 2. Fungal isolates used in this study to verify the specificity of PCR primers

Fungal species	No. of isolates	Origin	Year of isolation
Phaeomoniella chlamydospora	1	Toscana, Italy	
	2	Madrid, Spain	2003
	1	Ciudad Real, Spain	2003
Eutypa lata	1	Cataluña, Spain	2002
Acremonium alternata	2	Ciudad Real, Spain	2004
	2	Plant nursery in Valencia, Spain	2004
	1	Plant nursery in France	2004
Acremonium strictum	1	Ciudad Real, Spain	2004
Phialophora spp.	1	Plant nursery in Valencia, Spain	2004
Botryosphaeria obtusa	1	Ciudad Real, Spain	2004
	1	Plant nursery in Valencia, Spain	2004
Botryosphaeria parva	1	Plant nursery in Valencia, Spain	2004
Sporotrix spp.	1	Plant nursery in Valencia, Spain	2004
Phomopsis quercella	2	Plant nursery in Valencia, Spain	2004
Cylindrocarpon spp.	3	Plant nursery in Valencia, Spain	2004
	1	Ciudad Real, Spain	2004
Phomopsis spp.	1	Ciudad Real, Spain	2004
Phomopsis viticola	1	Plant nursery in Huesca, Spain	2004
Phomopsis spp.	1	Plant nursery in Valencia, Spain	2004
Phialophora mustea	1	Cataluña, Spain	2005
Phialemonium dimorphosporum	1	Cataluña, Spain	2005
Fomitiporia punctata	1	Cataluña, Spain	2005

obtained from the National Center for Biotechnology Information (NCBI) (Bethesda, MD). Nucleotide sequences were aligned using ClustalX version 1.81 (34) and then checked visually and adjusted manually if necessary. On the basis of these sequences, three putative primers were designed to amplify the ITS1 and ITS2 regions of rDNA (see Fig. 4). The primers were Pm1 (5′-CTC CAA ACC CTT TGT GAA CAT-3′) (forward primer), Pm2 (5′-CGA GCC CGC CAC TGA CTT-3′) (reverse primer), and Pm3 (5′-GCG AGC CCG CCA CTG ACT TT-3′) (reverse primer). The primers had no homology with other sequences as shown by a search done with the BLASTN program on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/). The Net Primer program (http://www.premierbiosoft.com/netprimer) was used to check the viability of these primers. Primers were synthesized by Sigma-Aldrich (Haverhill, Suffolk, United Kingdom).

PCR amplifications. PCR conditions were optimized using DNA from the seven *Phaeoacremonium* ex-type cultures for which primers were designed (Table 1). Then, the specificity of genus-specific primers was confirmed using DNA from all isolates listed in Tables 1 and 2. PCRs were carried out in a final volume of 25 μl, and the reaction mixtures contained 2 μl of 10× buffer [75 mM Tris HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$] (Biotools, Madrid, Spain), 0.2 μM (each) primer (Sigma-Aldrich, Haverhill, Suffolk, United Kingdom), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 1 U *Taq* polymerase (Biotools), and 10 ng of DNA template for each reaction. To achieve maximum efficiency in the amplification, 2.5 μl of bovine serum albumin (BSA) (10 mg/ml; Sigma-Aldrich, St. Louis, MO) and 10% of dimethyl sulfoxide (Amresco, OH) were added. DNA amplifications were carried out in a Perkin-Elmer 9700 thermal cycler (Applied Biosystems, Foster City, CA) by the following program: (i) an initial denaturation step at 94°C for 5 min; (ii) 40 cycles, with 1 cycle consisting of denaturation (30 s at 94°C), annealing (30 s at 52°C), and extension (50 s at 72°C); and (iii) a final extension step of 7 min at 72°C. The viability of DNA samples was checked with universal primers ITS1F and ITS4 at 53°C as the annealing temperature. Amplified fragments were visualized under UV light after electrophoresis on 1.5% agarose gels stained with ethidium bromide and run in 1× TBE buffer (Tris-borate-EDTA). A positive control (*Phaeoacremonium* ex-type DNA) and a negative control (no DNA) were included in each test.

Detection of *Phaeoacremonium* spp. in grapevine wood. A nested PCR was performed to achieve more sensitivity detecting *Phaeoacremonium* directly from wood. PCR conditions were optimized using DNA from the seven *Phaeoacremonium* ex-type cultures (Table 1), and then they were checked for specific amplification using the isolates listed in Table 2. A primary PCR was done using the universal primers ITS1F/ITS4 (14); it was performed in a volume of 25 μl containing 2 μl of 10× buffer, 0.2 μM (each) primer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 U of *Taq* polymerase, 2.5 μl of BSA, and 1 μl of DNA as template (approximately 10 ng of DNA). Conditions consisted of an initial denaturation step at 94°C for 2.5 min; 35 cycles, with 1 cycle consisting of 15 s at 94°C, 30 s at 53°C, and 90 s at 72°C; and a final extension step at 72°C for 7 min. PCR product was diluted 1:200, and then, 1 μl was used as DNA template for the secondary PCR using primer pair Pm1/Pm2. The concentrations of the reagents in a final volume of 25 μl were as follows: 2.5 μl of 10× buffer, 0.5 μM (each) primer, 4 mM $MgCl_2$, 0.8 mM dNTPs, and 1.25 U *Taq* polymerase. Thermal conditions were 5 min at 94°C, followed by 30 cycles (1 cycle consisting of 30 s at 94°C, 30 s at 57°C, and 50 s at 72°C), with a final extension step of 7 min at 72°C. DNA extracted from in vitro grapevine plants was included in all reactions as a negative control.

PCR detection of *Phaeoacremonium* spp. in wood was checked in nine grapevine plants sampled from a plant nursery. The sensitivity of PCR was compared to the sensitivity of the traditional method of culturing in a rich medium. Fragments of 3 to 4 cm long were taken from each plant (six or seven fragments from rootstock and one from each graft union and scion), and the bark was removed. Six wood sections (1 to 2 mm thick) were obtained from each fragment, and three fragments were used in each method. When disks were used to detect *Phaeoacremonium* by isolation in a growth medium, they were immersed in 70% ethanol for 1 min, air dried under sterile conditions, and plated on streptomycin-amended MEA (Conda Laboratories, Torrejón de Ardoz, Madrid, Spain) (three disks per plate). Petri dishes were incubated for 15 to 20 days at 25°C. Disks used for nested PCR were ground in liquid nitrogen, and DNA was extracted using the DNeasy plant mini kit. DNA samples were kept at −20°C until they were used for PCR amplifications.

Restriction enzyme digestion. Restriction maps of the ITS sequences were defined using the Mapdraw program from the Lasergene package (version 3.13; DNAstar, Madison, WI) for the holotype strains of nine *Phaeoacremonium* species. Three enzymes, BssKI, EcoO109I, and HhaI, that generated discriminant profiles among the species were selected. Conditions for enzyme digestion were as follows: (i) for BssKI, 3 units of enzyme (New England Biolabs, Beverly, MA), 2 μl of PCR product, 2 μl of the buffer enzyme, and 2 μl of BSA, digested for 1 hour at 60°C in a final volume of 20 μl; (ii) for EcoO109I, 1 unit of enzyme (Takara Bio Inc., Otsu, Shiga, Japan), 3 μl of PCR product, and 4 μl of the buffer enzyme, digested for 1 hour and 30 min at 37°C in a final volume of 40 μl; and (iii) for HhaI, 8 units of enzyme (Takara Bio Inc., Otsu, Shiga, Japan), 2 μl of PCR product, and 3 μl of the buffer enzyme, digested for 1 hour and 30 min at 37°C in a final volume of 30 μl. Restriction fragments were separated on 2.5% Metaphor agarose (Cambrex) using TBE buffer in the electrophoresis. An undigested PCR product was used as a control for nondigestion, and 100-bp (Biotools, Madrid, Spain) and 20-bp (Sigma-Aldrich, St. Louis, MO) molecular size markers were used to compare the size of each band. The digestion profile was visualized under UV light after staining with ethidium bromide.

Confirmation of PCR-RFLP method. To confirm that enzyme digestion patterns clearly differentiate species of *Phaeoacremonium*, the PCR-restriction fragment length polymorphism (RFLP) method was validated using CBS strains and several field isolates (Table 1). The species identifications of these field isolates were double checked by sequencing the ITS region amplified with universal primers ITS1F and ITS4 and by a subsequent BLAST search of the GenBank database (NCBI, Bethesda, MD).

β-Tubulin PCR. To distinguish *Phaeoacremonium angustius* from *Phaeoacremonium viticola*, PCR amplification using primers targeting the β-tubulin gene was done. Reverse primers Pbr4_1 and Pbr8 (25) were used to amplify *P. angustius* and *P. viticola*, respectively, in combination with universal forward primer T1 (26). PCR conditions were optimized using DNA from ex-type strains *P. angustius* (CBS114992) and *P. viticola* (CBS101738). PCR amplification was performed in a Perkin-Elmer 9700 thermal cycler (Applied Biosystems, Foster City, CA) as follows: (i) an initial denaturation step of 5 min at 94°C; (ii) five cycles, with one cycle consisting of denaturation (30 s at 94°C), annealing (30 s at 57°C), and extension (60 s at 72°C); (iii) five cycles, with one cycle consisting of denaturation (30 s at 94°C), annealing (30 s at 56°C), and extension (60 s at 72°C); and (iv) 25 cycles, with 1 cycle consisting of denaturation (30 s at 94°C), annealing (30 s at 55°C), and extension (60 s at 72°C); (v) a final extension step of 7 min at 72°C. PCR mix contained 10 ng of DNA template, 2.5 μl 10× buffer, 0.5 μM (each) primer (MWG-Biotech, Germany), 4 mM $MgCl_2$, 0.8 mM dNTPs, and 1.25 U *Taq* polymerase (Biotools, Madrid, Spain) in a final volume of 25 μl. These PCR conditions were tested using DNA from nine different species of *Phaeoacremonium* (Table 1) to confirm their specificity.

RESULTS

PCR detection of *Phaeoacremonium* spp. from mycelium. Amplifications performed with PCR primers Pm1 and Pm2

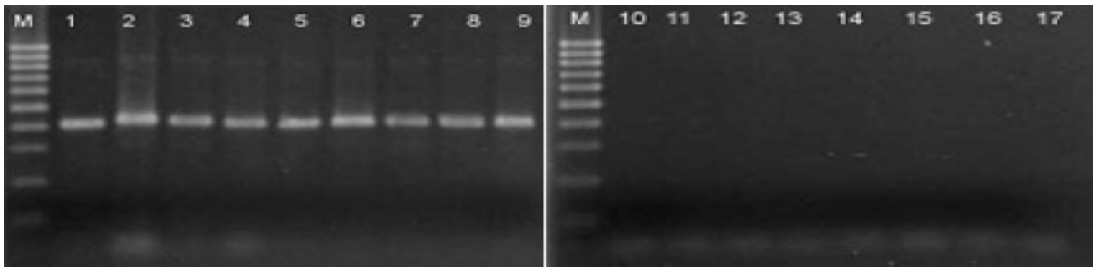

FIG. 1. PCR amplifications using primers Pm1 and Pm2 on DNA extracts from *Phaeoacremonium* (lanes 1 to 9) and other fungal species (lanes 10 to 15). Lanes: M, molecular size markers (100 bp); 1, *P. aleophilum* (CBS 246.91); 2, *P. parasiticum* (CBS860.73); 3, *P. inflatipes* (CBS391.71); 4, *P. mortoniae* (CBS101585); 5, *P. angustius* (CBS114992); 6, *P. viticola* (CBS101738); 7, *P. scolyti* (CBS113597); 8, *P. krajdenii* (CBS 109479); 9, *P. venezuelense* (CBS651.85); 10, *Botryosphaeria parva*; 11, *Phomopsis* spp.; 12, *Phaeomoniella chlamydospora*; 13, *Botryosphaeria obusa*; 14, *Phialophora mustea*; 15, *Phialemonium dimorphosporum*; 16, grapevine DNA; 17, no DNA.

yielded a fragment of the expected size (415 bp) for all seven ex-type strains of the *Phaeoacremonium* genus (Fig. 1). Furthermore, primer pair Pm1 and Pm2 detected more species than the seven *Phaeoacremonium* spp. for which the primers were initially designed (*P. aleophilum*, *P. parasiticum*, *P. rubrigenum*, *P. inflatipes*, *P. mortoniae*, *P. angustius*, and *P. viticola*). During the course of this work, new *Phaeoacremonium* species were defined, and DNA from some new *Phaeoacremonium* species tested (*P. krajdenii*, *P. venezuelense*, and *P. scolyti*) was successfully amplified using primers Pm1 and Pm2 (Fig. 1). Primer pair Pm1 and Pm3 yielded no PCR amplification for every *Phaeoacremonium* species tested, so they were not used to detect *Phaeoacremonium* species.

Designed primers Pm1 and Pm2 were evaluated using DNA from 49 *Phaeoacremonium* isolates (39 field isolates and 10 from CBS) (Table 1). An amplicon of approximately 415 bp was always obtained. In contrast, no amplification was obtained with DNA extracted from 28 isolates coming from different species isolated from grapevines (Table 2 and Fig. 1).

Detection of *Phaeoacremonium* spp. in grapevine wood. PCR amplifications using general fungal ITS primers were always successful and required a 200 times dilution of the PCR product to prevent inhibition in the secondary PCR. The final PCR product size was about 415 bp, as obtained in the simple PCR. Likewise, nested PCR specifically amplified *Phaeoacremonium* spp. but not any other fungal species listed in Table 2. DNA from in vitro grapevine plants was never amplified using primers ITS1F/ITS4.

Previous ground wood in liquid nitrogen allowed proper DNA extraction with the DNeasy plant mini kit. DNA extracted by this method was always visible on 0.8% agarose gel stained with ethidium bromide. The estimated DNA yield was about 100 ng/μl.

Nine plants were checked for the presence of *Phaeoacremonium* spp., and results were compared to those obtained incubating thin disks on MEA. The molecular method detected the pathogen in eight plants, whereas the traditional method of isolation detected it in five plants. The overall number of fragments per plant in which *Phaeoacremonium* was detected was higher by nested PCR (Table 3), so a lower number of fragments was required to detect an infected plant. When detection was performed by the traditional method of growing the fungus in a rich medium, the pathogen was found more frequently in the fragment below the graft union.

Identification of *Phaeoacremonium* species by PCR-RFLP. The three selected enzymes, BssKI, EcoO109I, and HhaI, digested PCR products successfully. *Phaeoacremonium* species were identified by the observed band profiles, although expected bands smaller than 40 bp were visualized with difficulty on a stained gel. Reaction conditions for each digestion were optimized using PCR amplicon DNA from nine *Phaeoacremonium* ex-types; once they were established, CBS strains and field *Phaeoacremonium* isolates previously classified by ITS sequences (Table 1) were used to confirm the results of this identification method. Finally, other *Phaeoacremonium* isolates (Table 1) were identified following the strategy explained below.

The first digestion was achieved with BssKI enzyme to distinguish *Phaeoacremonium aleophilum* from other *Phaeoacremonium* species. The BssKI-digested *Phaeoacremonium* amplicon showed a band of 338 bp for *P. aleophilum* and a band of 250 bp for other species (Fig. 2). This difference was sufficient to identify *P. aleophilum*. A second digestion was performed with EcoO109I to identify *P. parasiticum* and *P. scolyti*. The patterns were two bands of 344 bp and 49 bp for *P. parasiticum* and two bands of 288 bp and 56 bp for *P. scolyti*. These profiles were easily visualized in the stained gel (Fig. 2). The other species exhibited three different profiles: one that included *P. mortoniae* and *P. krajdenii* with bands of 340 bp and 72 bp; another group that included *P. venezuelense* and *P. inflatipes* with bands of 263 bp and 85 bp; and a last one with *P. angustius*

TABLE 3. Comparison of nested PCR and culturing methods to detect *Phaeoacremonium* spp. in fragments from naturally infected grapevine plants

No. of fragments/plant	No. of fragments with positive result[a] by:	
	Nested PCR	Culture in MEA
8	7	2
9	9	4
8	8	1
8	0	0
8	6	2
8	5	1
8	4	0
8	1	0
8	3	0

[a] Number of fragments in which *Phaeoacremonium* spp. were detected.

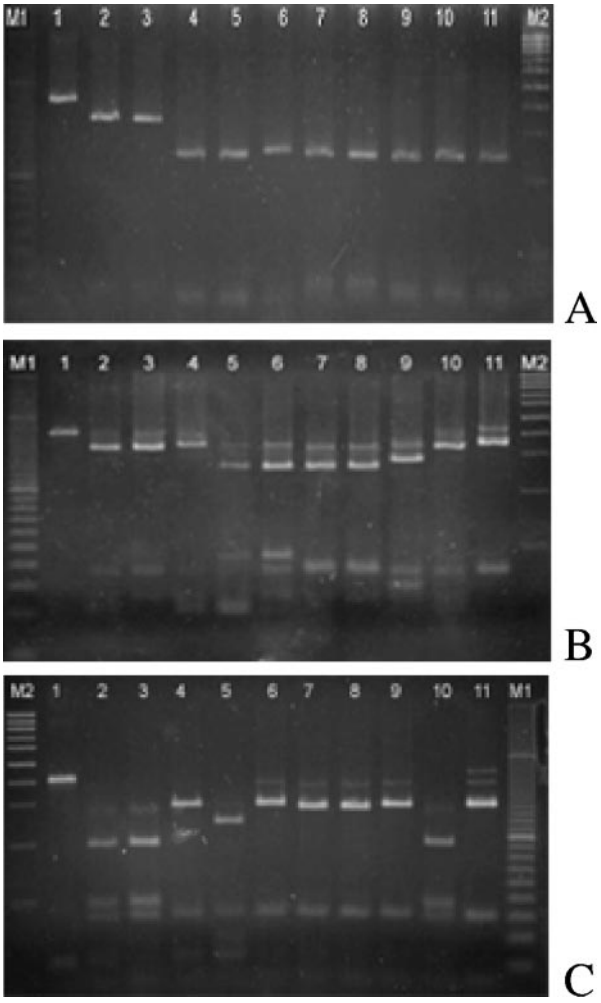

FIG. 2. Restriction fragment length polymorphism patterns of PCR-amplified *Phaeoacremonium* DNA using primers Pm1 and Pm2 digested with BssKI (A) Eco109I (B), and HhaI (C). Lanes: 1, undigested *Phaeoacremonium*; 2, *P. aleophilum* (CBS246.91); 3, *P. aleophilum* (CBS110753); 4, *P. parasiticum* (CBS860.73); 5, *P. inflatipes* (CBS391.71); 6, *P. venezuelense* (CBS651.85); 7, *P. viticola* (CBS101738); 8, *P. angustius* (CBS114992); 9, *P. scolyti* (CBS113597); 10, *P. mortoniae* (CBS101585); 11, *P. krajdenii* (CBS 109479); M1 and M2, molecular size markers of 100 bp and 20 bp, respectively.

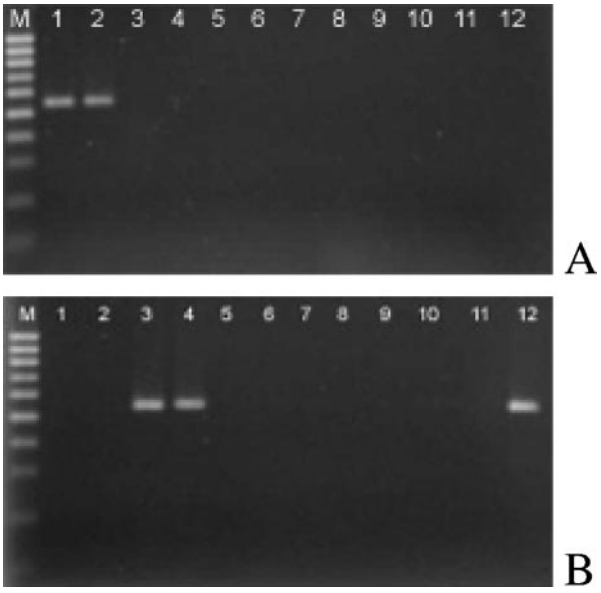

FIG. 3. PCR amplifications using species-specific primers T1/Pr4_1 (A) and primers T1/Pr8 (B) to identify *Phaeoacremonium angustius* and *Phaeoacremonium viticola*, respectively. Lanes: M, molecular size markers (100 bp); 1, *P. angustius* (CBS114992); 2, *P. angustius* (CBS 114991); 3, *P. viticola* (CBS101738); 4, *P. viticola* (CBS113065); 5, *P. parasiticum* (CBS860.73); 6, *P. aleophilum* (CBS 246.91); 7, *P. inflatipes* (CBS391.71); 8, *P. mortoniae* (CBS101585); 9, *P. krajdenii* (CBS 109479); 10, *P. venezuelense* (CBS651.85); 11, *P. scolyti* (CBS113597); 12, *P. viticola* (field isolate).

and *P. viticola* with bands of 262 bp and 71 bp. To sort out these species, a third digestion with HhaI enzyme was done. *P. venezuelense* was distinguished by a band of 296 bp, and *P. inflatipes* was distinguished by a 241-bp band. *P. mortoniae* showed a band of 192 bp, while *P. krajdenii* had a band of 297 bp. *P. angustius* and *P. viticola* were not distinguishable by digestion of the amplicon with any restriction enzyme, so it was necessary to use specific primers based on the β-tubulin gene to identify these species.

β-Tubulin PCR. PCR conditions were optimized for ex-type strains of *Phaeoacremonium angustius* and *Phaeoacremonium viticola* using primers targeting the β-tubulin gene (Pbr4_1/T1 and Pbr8/T1). Then, 16 CBS isolates (Table 1) were used to verify the specificity of these primers. Amplification of *P. angustius* DNA using primer pair Pbr4_1/T1 yielded a fragment of 556 bp, and amplification of *P. viticola* with Pbr8/T1 yielded a fragment of 542 bp. One field isolate that showed the band profile of *P. angustius*/*P. viticola*, was identified as *P. viticola* (Table 1) after specific PCR amplification using primers Pbr8/T1 (lane 12 in Fig. 3).

DISCUSSION

The objective of this work was to develop a PCR-based strategy to detect and identify *Phaeoacremonium* species isolated from grapevines. This method consists basically of a genus-specific amplification of the ITS1/5.8S/ITS2 region of rDNA and a further digestion to obtain a discriminant profile of the species. Nowadays, there is no other detection method described than the traditional one consisting of isolating fungi from a sample wood on a rich medium, even though the detection of *Phaeoacremonium* is important, especially for sanitation programs in grapevine nurseries. Primers (Pal1N/Pal2) for detection of *Phaeoacremonium aleophilum*, the most common species causing Petri disease, are available (33), but the sequences of these primers are highly similar to the sequences of other *Phaeoacremonium* species (Fig. 4). Primer Pal2 has complete homology with *P. mortoniae* and *P. angustius* sequences and high similarity with the remaining species; the sequence of primer Pal1N also is greatly similar to the sequences of other species of this genus. Moreover, some isolates included in the taxon of *P. aleophilum* were recently redefined as new species (*Phaeoacremonium alvesii* [CBS110034] and *Phaeoacremonium iranianum* [CBS101357]) (25). For these reasons, these primers are not specific for the detection of *P. aleophilum* and are useless in its identification. *Phaeoacremo-*

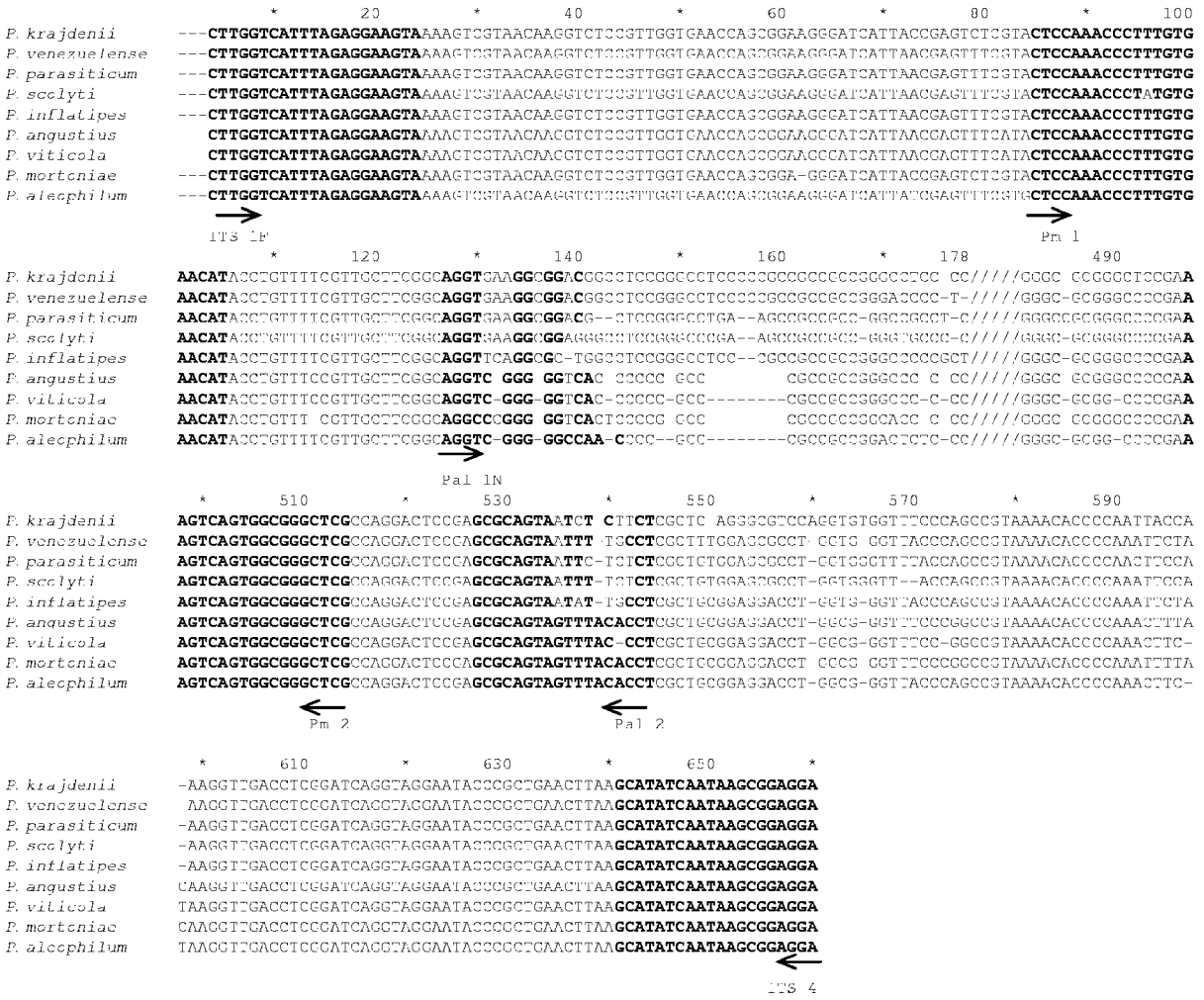

FIG. 4. rDNA sequence alignment of the ITS1/5.8S/ITS2 regions from species of *Phaeoacremonium* listed in Table 1. Dashes represent gaps, bold characters indicate the annealing sites for each primer, and slashes indicate that sequences from positions 178 to 480 are not provided because they are not necessary to illustrate alignment of sequences and primers. The sense of each primer is in accordance with the direction of the arrow shown below the sequences.

nium taxonomy has been extensively reviewed. In this study, 9 strains of 13 reported as growing on grapevines have been used. *P. iranium* and *P. austroafricanum* had not been described when this work was being carried out. *P. subulatum* is localized in South Africa, and *P. australiense* is found in Australia (25), and they were not included in this study. The method was evaluated for the remaining nine species (Table 1). DNA from each of the 38 isolates of *Phaeoacremonium* spp. was specifically amplified using the Pm1/Pm2 primer pair and it did not amplify DNA from other fungi collected from grapevines. More important, these primers recognized the new species of *Phaeoacremonium* defined when this work was being carried out (*P. scolyti*, *P. krajdenii*, and *P. venezuelense*). Therefore, these primers can be used to detect species of *Phaeoacremonium* infecting grapevines.

Two important advantages of using PCR for *Phaeoacremonium* detection are rapidity and sensitivity, qualities that are relevant when dealing with this pathogen. This fungus grows very slowly on enriched growth medium (25), which makes detection lengthy. This also means that *Phaeocremonium* is usually overgrown by other pathogens or saprobes, which may hide positive results of detection. Comparison of traditional and PCR methods to detect *Phaeocremonium* showed that the latter has greater sensitivity. Although the same amount of wood was used in a petri dish for fungal isolation and DNA extraction, PCR detected a higher number of infected fragments obtained from each plant (Table 3). Analysis was performed in 6 hours (extraction and PCR), while culturing and subsequent fungal isolation took up to 20 to 30 days and misidentification was not ruled out.

An effective measure to manage Petri disease is the use of pathogen-free plants in new vineyards (12, 32), and it is especially important, since it has been shown that infected plants are being used in new vineyards (1, 13, 15, 20). PCR amplifications carried out from wood extract are being performed to detect *Phaeomoniella chlamydospora* (16, 28, 33), the other important pathogen associated with Petri disease (4). It has been used to detect this pathogen in rootstocks and grapevine propagation plants (30), and it has shown the potential sources of *Phaeomoniella chlamydospora* in plant nurseries (29). Sim-

ilarly, implementation of the method presented here is suitable for production of *Phaeoacremonium*-free grapevine plants. The advantage of using designed, genus-specific primers is obvious, since there are several potential species of *Phaeoacremonium* occurring in a grapevine plant. If species-specific primers were to be used, a sanitation program for plant production could be much longer and expensive. Usage of the fungus-specific primer ITS1F (14) in the primary PCR prevents plant DNA amplification when detection is in wood.

All *Phaeoacremonium* species were unambiguously identified on the basis of their RFLP pattern, with the exception of *Phaeoacremonium angustius* and *Phaeoacremonium viticola*. However, when GenBank sequences of these two species (Table 1) were initially digested with a software application, results showed that it was possible that they were distinguished by digestion of the ITS region. Only after negative results in their differentiation were found, the sequences of ITS1F/ITS4-amplified region of *P. viticola* isolates (CBS101738 and CBS113065) were obtained, corroborating that the ITS sequences of *P. viticola* and *P. angustius* species exhibit 100% similarity. Similarity in ITS sequences was reported (17), which contributed to some misidentifications of these two species when *P. viticola* was defined as a new species (17). We conclude that the GenBank sequence of *P. viticola* (GenBank accession no. AF118137) is not right.

Taxonomy of *Phaeoacremonium* is difficult and slow. Basically, species are identified by cultural characters and morphology of conidia, conidiophores, and phialides. Since the genus was defined in 1996, new species have been included and described. Six species were originally included in the taxon that may be infecting grapevines, but this total number has nowadays become 13, which makes identification more difficult if possible. An alternative molecular method based on PCR amplification of DNA using species-specific primers in the β-tubulin gene has been recently designed (25), but a single PCR amplification is needed to identify each species. Restriction enzymes have been shown to be a powerful tool in the identification of *Phaeoacremonium* species by digestion of the ITS region and β-tubulin gene (9, 33). It was used before taxonomy of the genus was revised, allowing the identification of some species of *Phaeoacremonium*, in particular *P. parasiticum* and *P. inflatipes* (9). Digestion patterns also revealed that the former ex-type culture of *P. angustius* (CBS249.95) and *P. inflatipes* isolate (CBS222.95) were contaminated with *P. aleophilum* (9).

The strategy presented in this work has demonstrated to be robust enough for identification at the species level. Isolate CBS651.85 (type species of *Phaeoacremonium venezuelense* and formerly *Phaeoacremonium parasiticum*) was originally identified as *P. parasiticum* on the basis of its restriction profile when it was amplified with primers ITS4/ITS5 and digested with enzyme HhaI. This isolate has been used in this study, and it is clearly differentiated from *P. parasiticum* on the basis of RFLP patterns of Pm1/Pm2-amplified ITS region (Fig. 2).

The pathogenicity of *Phaeoacremonium* species is not yet fully established. Symptoms were reproduced by inoculation of *Phaeoacremonium aleophilum* (19, 21, 31), *P. inflatipes* (22), *P. krajdenii*, *P. parasiticum*, *P. subulatum*, *P. venezuelense*, and *P. viticola* (21). The other species were isolated from grapevines, but their pathogenicity has not been demonstrated. The identification of species becomes an important issue in disease management, especially when pathogenesis varies with the species. The method presented here is relatively simple compared to the traditional method that requires detailed observation of morphological characters and expertise evaluation of some characters, such as the type of phialides (24, 25). This method identifies all species of *Phaeoacremonium* in a maximum of three reaction mixtures. In summary, the PCR-based strategy presented here provides rapid, sensitive, and accurate detection and identification of *Phaeoacremonium* species in grapevines.

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