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Real-Time PCR Detection of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*

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***Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* are the two main fungal causal agents of Petri disease and esca. Both diseases cause significant economic losses to viticulturalists. Since no curative control measures are known, proactive defensive measures must be taken. An important aspect of current research is the development of sensitive and time-saving protocols for the detection and identification of these pathogens. Real-time PCR based on the amplification of specific sequences is now being used for the identification and quantification of many infective agents. The present work reports real-time PCR protocols for identification of *P. chlamydospora* and *P. aleophilum*. Specificity was demonstrated against purified DNA from 60 *P. chlamydospora* isolates or 61 *P. aleophilum* isolates, and no amplification was obtained with 54 nontarget DNAs. The limits of detection (i.e., DNA detectable in 95% of reactions) were around 100 fg for *P. chlamydospora* and 50 fg for *P. aleophilum*. Detection was specific and sensitive for *P. chlamydospora* and *P. aleophilum*. Spores of *P. chlamydospora* and *P. aleophilum* were detected without the need for DNA purification. The established protocols detected these fungi in wood samples after DNA purification. *P. chlamydospora* was detectable without DNA purification and isolation in 67% of reactions. The detection of these pathogens in wood samples has great potential for use in pathogen-free certification schemes.**

Phaeoacremonium species and *Phaeomoniella chlamydospora* are the main fungal causal agents of Petri disease and esca (24). *Phaeoacremonium* spp. were associated especially with grapevine diseases and disease symptoms of a number of woody hosts, including oak (*Quercus* sp.), ash (*Fraxinus* sp.), willow (*Salix* sp.), olive (*Olea europea*), apricot (*Prunus armeniaca*), cherry (*Prunus avium*), kiwifruit (*Actinidia* sp.), and date palm (*Phoenix dactylifera*) trees (8, 9, 11, 16, 20, 23) as well as being associated with human infections called phaeohyphomycoses (defined as tissue invasion by fungi with melanized cell walls) (18). *Phaeoacremonium* spp. have a worldwide distribution. Since 1996, the number of known species associated with Petri and esca disease has increased quickly (8, 10, 13, 16). To date, 34 species of the anamorph genus *Phaeoacremonium* have been described, of which 12 were linked to *Togninia* teleomorphs (9, 23). The molecular characteristics of the β-tubulin and actin genes have played an important role in the detection and identification of *Phaeoacremonium* spp. (23). The occurrence of *Phaeoacremonium* spp. varies among grape-growing countries, with *P. aleophilum* the species most frequently found to affect grapevines worldwide (23, 13, 22). It was suggested that this pathogen disseminated from one host to another, such as kiwifruit and *Prunus* sp. (9). *P. chlamydospora* is a haploid ascomycete fungus, and no teleomorph is known. Several genetic diversity studies have reported limited variation and support the presumed predominance of asexual reproduction (4, 5, 6, 27).

Petri disease affects mostly 1- to 5-year-old grapevines. The external symptoms include stunted growth, interveinal chlorosis, and decline. Dissected vines show brown xylem vessels, the result of tyloses, gums, and phenolic compounds being formed by the host in response to infection (24). Blocked xylem vessels accentuate water stress and lead to insufficient water and nutrient supplies to the vegetative parts of the plant. These, plus the production of phytotoxic metabolites (32), contribute to other symptoms appearing during periods of high water demand. The presence of symptoms in young vines supports the assumption that propagation provides a means for the fungus to spread (11, 17, 19). Esca is a complex disease associated with at least three species of fungi: *P. chlamydospora*, *P. aleophilum*, and *Fomitiporia mediterranea* (32). The disease affects mature vines and is associated with symptoms that can range from mild to severe and chronic. The severe form is characterized by a sudden wilting of the leaves in all parts of the infected vine and the shriveling of all fruit. Berry symptoms in chronically affected plants appear as superficial brown to purple spots scattered over the surface. Leaf symptoms involve interveinal chlorosis, as described for Petri disease. On the trunk, dark brown streaks appear along with pink-brown areas on the margin of necrotic tissues; white rot also occurs, which reduces the wood to a light-colored spongy mass. Petri disease and esca are grapevine diseases of worldwide importance. Outbreaks have been reported from France (20), California (31), Italy (27), Portugal (28), New Zealand (30), South Africa (19), Australia (11), and Spain (22).

The importance of grapevines to the economies of Spain, France, Italy, and other countries plus the recurrence of Petri disease and esca in recent years have stimulated research into these complex diseases. An improvement in the detection and identification of the causal pathogens would be an important step forward. The conventional methods used to identify *P. chlamydospora* and/or *P. aleophilum* in grapevines involve a combination of recognition of symptoms, fungal isolation, and morphological examination of fungi able to grow under laboratory conditions. However, symptoms can be unreliable, with some plants appear-

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ing asymptomatic for long periods, and distinguishing species based on their morphology is time-consuming and generally requires the destruction of part of the vines. There is therefore a need for an accurate, rapid, and affordable test to detect Petri disease and esca fungi based on molecular methods, such as PCR. Conventional PCR has been successfully used for detection of *P. chlamydospora* (33). A nested-PCR protocol for detecting species of *Phaeoacremonium* in plants also exists (2). Quantitative real-time SYBR green PCR techniques have also been investigated for the detection of *P. chlamydospora* and *Phaeoacremonium* spp. (26), although the primers and probe used were based on the internal transcribed spacer (ITS) region; this is now well known not to differentiate between *Phaeoacremonium* species. Edwards et al. (12) compared conventional PCR, nested-PCR, SYBR green PCR, and TaqMan PCR methods and concluded that nested-PCR and TaqMan PCR were the most sensitive methods for detecting *P. chlamydospora*, although further research was required to optimize their use.

The aim of the present study was to develop a rapid, sensitive, and specific method for the detection of *P. chlamydospora* and *P. aleophilum* using real-time PCR. These two species represent around 16 to 18% of the 30 species associated with grapevine decay in Castilla y León, Spain (22). Once the PCR protocols were established with purified DNA from isolated fungi, as is usually done, attempts were made to detect these fungi in spore suspensions without DNA purification. Then to speed up the methodology, efforts were undertaken to eliminate the fungi cultivation. Isolation and identification steps were performed using directly infected wood chips, and then DNA purifications of such samples were assayed. Finally, to reduce the manipulation much more, wood chips were incubated in liquid medium, and this liquid was used as the DNA template in the TaqMan PCR.

MATERIALS AND METHODS

Fungal strains, culture media, and growth conditions. Fungi were isolated from branches of adult grapevines and complete young vines affected with Petri disease, esca, or other types of grapevine decay. These were mainly collected in the Spanish region Castilla y León. This study included 60 *P. chlamydospora* and 61 *P. aleophilum* isolates. Sixty-one isolates of other species were used as controls. Fifteen grapevines gave both *P. chlamydospora* and *P. aleophilum* isolates. For example, grape Y38 gave Y38-8-1c (*P. chlamydospora*) and Y38-5-3a (*P. aleophilum*). Twenty-five cultures were obtained from the Centraalbureau voor Schimmelcultures (CBS Fungal Collection, Utrecht, The Netherlands). Tables S1, S2, and S3 in the supplemental material show the isolates' names and where they were obtained. All isolates were grown on potato dextrose agar (PDA) (Merck, Darmstadt, Germany) at 25°C in darkness. Mycelia were lyophilized and stored at −20°C.

DNA purification. Around 60 mg of lyophilized mycelia was ground to a fine powder in liquid nitrogen using a mortar and pestle. Genomic DNA was isolated using the DNeasy plant minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA quantity and quality were determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). DNA was amplified with the ITS5 and ITS4 primers (34) and/or Pch1 and Pch2 or Pal1N and Pal2 (33) to ensure the presence of amplifiable DNA.

Primers and probe design. DNA from 35 *P. chlamydospora* isolates (see Table S1 in the supplemental material) was amplified using primers ITS4 and ITS5. PCR fragments (about 650 bp) were purified with the Illustra GFX PCR DNA and gel band purification kit (Amersham Biosciences, GE Healthcare, United Kingdom) and sequenced by the Instrumental Techniques Laboratory of the University of León (Spain). The DYEnamic ET dye terminator kit (MegaBACE, Amersham Biosciences) was used for the sequencing reaction; amplification was performed using an MJ Research PTC-200 thermocycler (Amersham Biosciences). The DNA sequences obtained were analyzed with a MegaBACE 500 sequencer. The completed sequences of the ITS region were read and edited using Chromas v. 1.45 software (Southport, Australia). Multiple alignments of the 529-bp sequences were carried out with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/#) and showed only 2 bases that differed at positions 369 and 438, grouping the 35 isolates into two groups: group I (14 isolates) with bases T369 and T438 and group II (21 isolates) with bases A369 and C438 (6). Three sequences of each group were selected and deposited in NCBI under accession no. EU018412, EU018413, EU018414, EU018415, EU018416, and EU018417, respectively (6). The selection also considered the origin and the external symptom of the grapevine samples collected in Castilla y León. Consensus sequence was obtained from these sequences plus seven others (AF017652; AF266653; AF266656; AF266652; AF197987, and AF197986) from the GenBank database (National Center for Biotechnology Information [NCBI], Bethesda, MD).

DNA of 22 *P. aleophilum* isolates (see Table S2 in the supplemental material) was amplified using primers T1 (25) and Bt2b (15) of the β-tubulin gene. PCR fragments (about 650 bp) were purified, sequenced, and analyzed as described above. Five sequences were deposited in NCBI under accession no. JF275874, JF285876, JF275879, JF275877, and JF275878, respectively. They showed bases that differed at position 96, 365, or 464. The consensus sequence of *P. aleophilum* was obtained (652 bp) from these sequences plus eight others from NCBI (DQ173095, DQ173094, EU863466, EU863469, EU863472, EU863468, EU863467, and EU863470). The sequences of *P. aleophilum* and other species (*P. scolyti*, AY579293, EU128091, EU128088, EU260415, and AY579292; *P. viticola*, DQ173105, DQ173107, AF192391, and AF246816; *P. hispanicum*, HQ700718; *P. iranianum*, DQ173096, DQ173097, and DQ173098; and *P. mortoniae*, DQ173109, EF517921, EU863480, and EU863475) of the genera obtained from GenBank were used for the design of the primers.

Primers and probes were designed using Primer Express v.2.0 software (Applied Biosystems, Foster City, CA) and synthesized by MWG-Biotech AG (Ebensburg, Germany). Figure S1 in the supplemental material presents the sequences and the locality of the primers and probe designed in the ITS2 region and in the β-tubulin gene. TaqMan probes for *P. chlamydospora* and *P. aleophilum* were labeled at the 5′ end with the fluorophores 6-carboxyfluorescein (FAM) and hexachlorofluorescein (HEX), respectively. Both probes were labeled on the 3′ end with the black hole quencher 1 (BHQ1) dye.

PCR conditions. (i) SYBR green PCR. Reactions were performed in a final reaction volume of 20 μl containing Power SYBR-Green 1× PCR master mix (Applied Biosystems), 0.3 μM each primer, and 5 μl of DNA sample at different concentrations. Reactions were run in a 7500 real-time PCR system platform (Applied Biosystems) using the following standard program: 10 min at 95°C plus 50 cycles of 15 s at 95°C and 1 min at 60°C.

(ii) TaqMan PCR. PCR conditions and reagent concentrations were optimized to obtain the final parameters described below. Reactions were performed in 20-μl reaction volumes containing 1× TaqMan Universal PCR master mix (Applied Biosystems), 0.3 μM each primer, 0.15 μM TaqMan probe, and 5 μl of DNA template. In all PCR experiments, a positive control (*P. chlamydospora* CBS239.74 or *P. aleophilum* Y161-19-2a) and nontemplate control (NTC; water) were included to test PCR performance. Amplification products were analyzed using Sequence Detection System software v.1.2.3 (Applied Biosystems).

The efficiency of the *P. chlamydospora* and *P. aleophilum* TaqMan PCR systems designed was calculated following the recommendations of Bustin et al. (5). The TaqMan PCR efficiency for each system was calculated taking into account the two different templates: purified genomic DNA and suspension of spores obtained from the same strain. The 10-fold serial dilutions from genomic DNA or the spore suspensions of *P. chlamydospora* and *P. aleophilum* were prepared from concentrated samples to obtain a broad range of dilutions. Each dilution was analyzed with the *P.*

TABLE 1 Determination of the detection limits of the TaqMan PCR assay with genomic DNA from *P. chlamydospora* CBS239.74

fg/reaction	C_T value[a]	Signal ratio[b]
108,000,000	15.51 ± 0.29	9/9
10,800,000	17.38 ± 0.05	9/9
1,080,000	20.90 ± 0.05	9/9
108,000	24.51 ± 0.09	9/9
10,800	27.81 ± 0.05	9/9
1,080	31.92 ± 0.27	9/9
108	35.73 ± 0.48	9/9
54	36.81 ± 0.89	24/30
22	37.58 ± 0.99	18/30
11	37.42 ± 0.71	17/30
1	37.26	1/30

[a] Cycle number at which fluorescence intensity equals a fixed threshold. Shown are mean values ± standard errors of the means. The results are the means of three independent experiments with three replicates each (first seven dilutions) or three independent experiments with 10 replicates each (last four dilutions). The linearity (R^2) was 0.9988.
[b] Number of positive results out of the total number of reactions.

TABLE 3 Determination of the detection limits of the TaqMan PCR assay with spores from *P. chlamydospora* CBS239.74

No. of *P. chlamydospora* spores	C_T value[a]	Signal ratio[b]
28,000	25.48 ± 0.11	9/9
2,800	29.03 ± 0.07	9/9
280	32.98 ± 0.26	9/9
100	34.57 ± 0.38	9/9
50	35.41 ± 0.23	9/9
20	37.14 ± 0.07	27/30
10	37.26 ± 0.20	19/30
5	37.33 ± 0.30	6/30
1	39.58	1/30

[a] Cycle number at which fluorescence intensity equals a fixed threshold. Shown are mean values ± standard errors of the means. The results are the means of three independent experiments with three replicates each (first five dilutions) or three independent experiments with 10 replicates each (last four dilutions). The linearity (R^2) was 0.9944.
[b] Number of positive results out of the total number of reactions.

chlamydospora and *P. aleophilum* TaqMan PCR assays in triplicate with 5 and 7 different isolates, respectively. *P. chlamydospora* CBS239.74 and *P. aleophilum* Y161-19-2a were analyzed to complete the TaqMan PCR assays with more repetitions of the lower dilutions corresponding to 54, 22, 11, and 1 fg DNA per reaction and 50, 25, 5, and 1 fg DNA per reaction, respectively. These dilutions were assayed 30-fold, as indicated in Tables 1 and 2. Similar dilution procedures were followed with spore studies (Tables 3 and 4). The serial dilutions of genomic DNA or spores were used to develop three TaqMan PCR assays that were made in different run times. The mean values of the threshold cycle (C_T) obtained were plotted against the logarithm of the DNA concentration in the reaction in order to construct the standard curve. The slope of the standard curve was used to calculate PCR efficiency, applying the equation $E = 10^{-1/\text{slope}} - 1$ (5). In the same way, the *P. chlamydospora* and *P. aleophilum* TaqMan PCR systems were tested with a suspension of spores of *P. chlamydospora* CBS239.74 and *P. aleophilum* Y161-19-2a to confirm the development of the TaqMan PCR system using a suspension of spores as the template in the TaqMan PCR. The PCR efficiency was calculated as for the genomic DNA template.

***P. chlamydospora* and *P. aleophilum* spore preparation.** *P. chlamydospora* and *P. aleophilum* spores were obtained from 1 to 2 cm of fungal mycelium grown on potato dextrose agar (PDA) (collected after approximately 1 month of growth). PDA plates were agitated with 1 ml of Milli-Q water, and the latter was sucked into and blown out of a pipette under sterile conditions to liberate the spores, which were then transferred to a tube for later counting using a Neubauer chamber (BlauBrand, Germany) or used as a DNA template (5 μl) in the TaqMan PCR. Spore concentrations were determined using the formula spores/ml = $N \times 25 \times 104$, with N the average spore number counted in each cell.

***P. chlamydospora* and *P. aleophilum* detection in infected wood samples.** Twelve *Vitis vinifera* (cv. Tempranillo grafted onto 110R rootstocks) vines were inoculated with each of the *P. chlamydospora* isolates Y121-29-6c, Y116-18-3c, or Y106-4-3b, and 12 vines were inoculated with each of the *P. aleophilum* isolates Y108-2-2z, Y38-5-3z, or Y235-4-1w. Twelve control plants were inoculated with a sterile agar plug (PDA). For the inoculation, a wound was produced in the trunk of the vine, and an agar plug containing or not an actively growing culture of each isolates was placed on the wound and covered with Parafilm. All grapevines were maintained in a greenhouse at 20 to 25°C. After 4 months, the *P. chlamydospora* or *P. aleophilum* strain was reisolated and identified. Our experience demonstrated that these fungi could be reisolated 1 cm over the inoculation point, and incipient wood symptoms could be observed. The effectiveness of the inoculation was evaluated using the conventional method of fungus isolation (22), consisting of cutting six wood chips (approximate diameter of 1 to 2 mm and approximate length of 0.5 to 1 cm) that were placed on malt extract agar (MEA) plates (Merck, Darmstadt, Germany) and incubated at 25°C in darkness until fungi grew to a size at which they could be isolated and placed on PDA plates. As de-

TABLE 2 Determination of the detection limits for the TaqMan PCR assay with genomic DNA from *P. aleophilum* Y161-19-2a

fg/reaction	CT value[a]	Signal ratio[b]
5,000,000	22.00 ± 0.91	9/9
500,000	25.73 ± 0.91	9/9
50,000	29.22 ± 1.01	9/9
5,000	32.67 ± 1.54	9/9
500	36.17 ± 1.46	9/9
50	38.92 ± 1.06	29/30
25	38.90 ± 1.78	9/30
5	40.89 ± 0.98	7/30
1	Undetectable	0/30

[a] Cycle number at which fluorescence intensity equals a fixed threshold. Shown are mean values ± standard errors of the means. The results are the means of three independent experiments with three replicates each (first five dilutions) or three independent experiments with 10 replicates each (last four dilutions). The linearity (R^2) was 0.9974.
[b] Number of positive results out of the total number of reactions.

TABLE 4 Determination of the detection limits of the TaqMan PCR assay with spores from *P. aleophilum* Y161-19-2a

No. of *P. aleophilum* spores	C_T value[a]	Signal ratio[b]
30,000	25.92 ± 0.54	9/9
3,000	29.65 ± 0.63	9/9
300	32.87 ± 0.52	9/9
30	36.14 ± 0.68	29/30
15	37.19 ± 0.97	24/30
3	40.98 ± 2.11	21/30
1	Undetectable	0/30

[a] Cycle number at which fluorescence intensity equals a fixed threshold. Shown are mean values ± standard errors of the means. The results are the means of three independent experiments with three replicates each (first three dilutions) or three independent experiments with 10 replicates each (last four dilutions). The linearity (R^2) was 0.9879.
[b] Number of positive results out of total number of reactions.

scribed above, the PDA plates were incubated and the colonies that grew were morphologically and/or molecularly identified. The inoculated plants used in this study correspond to plants in which the inoculated fungi were isolated and identified from the six wood chips placed on MEA. Three wood chips were cut from four control plants and from four plants infected with each isolate. DNA purification was performed with one chip of each grapevine using the Qiagen kit as described above. The second chip from each was placed in a tube containing 150 μl of malt extract (ME) medium, and the third chip was incubated in 150 μl sterile water. After 2 days at 25°C in darkness, TaqMan PCR was performed under the conditions described above with 5 μl of purified DNA, 5 μl of the incubation ME, or 5 μl of the incubation water.

Nucleotide sequence accession numbers. Five new sequences of *P. aleophilum* isolates have been deposited in the NCBI database under accession no. JF275874, JF285876, JF275879, JF275877, and JF275878.

RESULTS

Selection of primer sets. For the design of the primers and probe for *P. chlamydospora*, the DNA of 35 isolates was amplified using primers ITS4 and ITS5. A consensus sequence was obtained (528 bp) from these sequences plus seven others from NCBI. Two primer pairs were designed: one based on the ITS1 fragment and one based on the ITS2 fragment. Primer viability was tested in the SYBR green system with 60 *P. chlamydospora* isolates plus those of 16 other species (see Tables S1 and S3 in the supplemental material). The primer pair based on ITS1 amplified all target *P. chlamydospora* DNAs but also produced nonspecific reactions with nontargeted DNAs. The second primer pair amplified only the target DNA of *P. chlamydospora* (data not shown) and was therefore selected to be used in the TaqMan PCR system. The TaqMan probe pchITS439P was then designed based on the ITS2 fragment (primers pchITS417F and pchITS495R, generating a fragment of 78 bp [see Fig. S1 in the supplemental material]).

The design of the primers and probe for *P. aleophilum* was based on the alignment of β-tubulin gene sequences. The DNAs of 22 isolates of *P. aleophilum* were amplified using primers T1 and Bt2b, generating a sequence beginning at exon 2 and ending at exon 6 of the β-tubulin gene. A consensus sequence of 652 bp was obtained from the sequences of the 22 isolates plus others from the GenBank database. Alignment of the DNA sequences of *P. aleophilum*, *P. scolyti*, *P. viticola*, *P. hispanicum*, *P. iranianum*, and *P. mortoniae* allowed primer design. Three primer pairs were designed: one based on intron 1, one based on intron 1 to intron 2, and one based on intron 3 to intron 4. Primer viability was tested in the SYBR green PCR system with 22 *P. aleophilum* isolates plus those of 23 other species (see Tables S2 and S3 in the supplemental material). Only the primers based on intron 1 (primers palFI1 and palRI1, producing a fragment of 63 bp) amplified all 22 target DNAs. Thus, primer pair palFI1/palRI1 was selected to be used in the real-time PCR system, and the TaqMan probe palI1 was designed based on intron 1 (see Fig. S1 in the supplemental material). The two selected primer pairs yielded single amplicons of 78 bp and 63 bp, respectively, for *P. chlamydospora* and *P. aleophilum*.

Specificity. The selected systems were experimentally tested with purified DNA. The amplifiability of the DNA samples was checked by conventional PCR using ITS5 and ITS4 primers and/or the Pch1 and Pch2 or the Pal1N and Pal2. All fungus samples showed positive amplification, showing they were suitable for TaqMan PCR assays. The *P. chlamydospora* TaqMan PCR system successfully amplified the target DNA templates of 60 isolates of *P. chlamydospora* (see Table S1 in the supplemental material). No amplification was obtained with 54 nontarget DNA templates (see Table S3 in the supplemental material). The method was 100% specific. The *P. aleophilum* TaqMan PCR system successfully amplified the target DNA templates of 61 *P. aleophilum* isolates (see Table S2 in the supplemental material). No amplification was obtained with 54 non-*P. aleophilum* DNAs (see Table S3 in the supplemental material). The method was 100% specific.

Sensitivity. The sensitivity of the TaqMan PCR assays was evaluated for *P. chlamydospora* CBS239.74 and for *P. aleophilum* Y161-19-2a isolates using purified DNA from mycelia of isolated fungi. Spore suspensions were also used directly as templates without any DNA extraction or purification. Assays were performed as three independent experiments with nine replicates for each dilution, except those containing <108 fg/reaction of *P. chlamydospora* DNA, <500 fg/reaction of *P. aleophilum* DNA, or <50 spores for *P. chlamydospora* or <300 spores for *P. aleophilum* (30 replicates). The following dilutions of *P. chlamydospora* DNA were used as templates: 108×10^6, 108×10^5, 108×10^4, 108×10^3, 108×10^2, 1,080, 108, 54, 22, 11, and 1 fg/reaction. The results obtained (Table 1) allowed standard curves to be plotted, defined by the equation $y = -3.6282x + 42.8190$, with a regression coefficient of close to 1 ($R^2 = 0.9988$). This indicates a highly linear reaction and a system efficiency of 0.886. The limit of detection (LOD) was defined as the minimum level at which the analyte could be reliably detected with a probability of 95% (108 fg of DNA/reaction). However, the TaqMan PCR assay was able to detect about 10 fg/reaction of *P. chlamydospora* DNA in 57% of reactions.

The following dilutions of *P. aleophilum* DNA were used as templates: 500×10^4, 500×10^3, 500×10^2, 5,000, 500, 50, 25, 5, and 1 fg/reaction. The results obtained (Table 2) allowed standard curves to be constructed as above, defined by the equation $y = -3.3166x + 44.5520$, a regression coefficient ($R^2 = 0.9974$), and a system efficiency of 1.002. The LOD was established at 50 fg/reaction. The TaqMan PCR assay was able to detect 5 fg/reaction of *P. aleophilum* DNA in 23% of reactions.

The following dilutions of *P. chlamydospora* spore suspension were tested without any DNA extraction: 28,000, 2,800, 280, 100, 50, 20, 10, 5, and 1 spore per reaction. The dilution curve for the number of *P. chlamydospora* spores was defined by the equation $y = -3.5622x + 41.437$, an R^2 value of 0.9944, and a system efficiency of 0.909. The LOD was 50 spores. However, positive amplification was obtained with 10 spores in 63% of reactions (Table 3).

The following dilutions of *P. aleophilum* spore suspension were tested without any DNA extraction: 30,000, 3,000, 300, 30, 15, 3, and 1 spore per reaction. The dilution curve for the number of *P. aleophilum* spores was defined by the equation $y = -3.6247x + 42.0951$, an R^2 value of 0.9879, and a system efficiency of 0.926. The LOD was 30 spores. However, positive amplification was obtained with three spores in 70% of reactions (Table 4).

Complementation of the spore assay using further strains. Spores from 10 different isolates of *P. chlamydospora* (LR9a, INIA56b, V13-1a, S2-1-1, S5-3-1, Y38-8-1c, Y80-6-1b, Y105-3-2a, Y119-8-5b, and Y162-20-1) and 10 other isolates of *P. aleophilum* (CBS631.94, CLM51.6b, S4-3-1, Y38-5-3a, Y107-6-1b, Y179-17-1b, Y213-16-5a, Y230-3-2b, Y233-5-4b, and Y272-4-1) were obtained directly from PDA cultures (5 μl of liberated spore sus-

TABLE 5 Detection of *P. chlamydospora* in wood chips

Treatment of wood chips[a]	C_T value with[b]: Purified DNA	2 days of incubation: ME medium	2 days of incubation: Water
Inoculation with isolate:			
Y121-29-6c-5e	17.94 ± 0.04	28.0 ± 1.19	35.6 ± 2.15
Y121-29-6c-8e	18.18 ± 0.01	30.33 ± 0.15	29.68 ± 0.57
Y121-29-6c-9e	18.22 ± 0.04	31.32 ± 0.17	Undetectable
Y121-29-6c-12e	22.20 ± 0.06	34.37 ± 2.63	32.23 ± 0.21
Y116-18-3c-2	24.60 ± 0.03	Undetectable	Undetectable
Y116-18-3c-4e	23.97 ± 0.04	36.21 ± 0.37	30.6 ± 5.44
Y116-18-3c-7e	23.34 ± 0.04	29.91 ± 0.36	23.65 ± 5.45
Y116-18-3c-11e	19.67 ± 0.10	25.53 ± 0.27	Undetectable
Y106-4-3b-3	26.76 ± 0.02	Undetectable	Undetectable
Y106-4-3b-4	24.11 ± 0.05	42.21 ± 9.18	Undetectable
Y106-4-3b-8	25.63 ± 0.02	Undetectable	Undetectable
Y106-4-3b-11	25.09 ± 0.04	Undetectable	Undetectable
Sterile PDA (control)	Undetectable	Undetectable	Undetectable

[a] Shown are TaqMan PCR results with the pchITS417F/pchITS495R-pchITS439P system for wood samples inoculated with different isolates of *P. chlamydospora* and for control wood treated with sterile PDA.
[b] Cycle number at which fluorescence intensity equals a fixed threshold. Shown are mean values ± standard errors of the means. The results obtained represent three replicates. The percentages of successful reactions with purified DNA, ME medium, and water were 100%, 67%, and 42%, respectively.

TABLE 6 Detection of *P. aleophilum* in wood chips

Treatment of wood chips[a]	C_T value with[b]: Purified DNA	2 days of incubation: ME medium	2 days of incubation: Water
Inoculation with isolate:			
Y108-2-2z-1	26.93 ± 0.26	Undetectable	Undetectable
Y108-2-2z-2	25.42 ± 0.13	34.12 ± 2.94	Undetectable
Y108-2-2z-4	29.29 ± 0.37	Undetectable	Undetectable
Y108-2-2z-5	28.94 ± 0.63	Undetectable	Undetectable
Y38-5-3z-4	31.08 ± 0.47	Undetectable	Undetectable
Y38-5-3z-5	25.11 ± 0.26	Undetectable	Undetectable
Y38-5-3z-6	31.27 ± 0.25	33.86 ± 0.60	Undetectable
Y38-5-3z-7	32.87 ± 0.37	34.23 ± 1.48	Undetectable
Y235-4-1w-1	34.00 ± 0.40	Undetectable	Undetectable
Y235-4-1w-3	26.02 ± 0.03	Undetectable	Undetectable
Y235-4-1w-5	26.72 ± 0.27	Undetectable	Undetectable
Y235-4-1w-7	32.52 ± 0.58	Undetectable	Undetectable
Sterile PDA (control)	Undetectable	Undetectable	Undetectable

[a] Shown are TaqMan PCR results with the palFI1/palRI1-palI1 system for wood samples inoculated with different isolates of *P. aleophilum* and for control wood treated with sterile PDA.
[b] Cycle number at which fluorescence intensity equals a fixed threshold. Shown are mean values ± standard errors of the means. The results obtained represent three replicates. The percentages of successful reactions with purified DNA, ME medium, and water were 100%, 25%, and 0%, respectively.

pensions). All were successfully detected with the respective TaqMan PCR systems described above (data not shown).

Detection of *P. chlamydospora* and *P. aleophilum* in wood samples. DNA purified from wood chips obtained from the grapevines inoculated with three different *P. chlamydospora* isolates showed C_T values ranging from 17.94 ± 0.04 to 26.67 ± 0.02 for all samples. No amplification was observed with wood inoculated with a PDA plug containing no mycelium (Table 5). DNA purified from wood chips obtained from grapevines infected with three *P. aleophilum* isolates showed C_T values ranging from 25.11 ± 0.26 to 34.00 ± 0.40 in 100% of reactions (Table 6); no amplification was obtained with the control vine. These results confirmed the above identification done using conventional methods consisting of the culturing of six wood chips in culture medium for each inoculated plant and morphological identification of the isolated fungi.

Positive amplification of *P. chlamydospora* in 67% of reactions was obtained with wood chips incubated for 2 days in culture medium with no DNA purification (Table 5). The four grapevines inoculated with the Y121-29-6c isolate returned C_T values ranging from 28.0 ± 1.19 to 34.37 ± 2.63, while only one sample of the four grapevines inoculated with Y106-4-3b was positive, as were three samples of the four grapevines inoculated with Y116-18-3c. When incubation was performed in water for 2 days, the amplification results were lower (positive detection in 42% of reactions), with better detection in the plant inoculated with *P. chlamydospora* Y121-29-6c and Y116-18-3c. Positive amplification of *P. aleophilum* DNA, with no DNA purification, was obtained with wood chips incubated for 2 days in culture medium in only 25% of reactions (Table 6). No amplification was seen when samples were incubated in water for 2 days. Isolate Y38-5-3z was detected in two of the four inoculated plants after incubation, while isolate Y235-4-1w was not detected. *P. chlamydospora* and *P. aleophilum* were 100% successfully detected in wood chips after DNA purification as was also found with the conventional method. At least for the detection of *P. chlamydospora*, the incubation in culture medium could be used as a template without the need for DNA purification and fungus isolation.

DISCUSSION

The correct, fast, and sensitive identification of fungi involved in Petri disease and esca is a crucial precondition for the conduct of meaningful studies on the epidemiology of these species associated with destructive diseases of grapevines and other species. For this purpose, the most appropriate method is the use of DNA-based tools, such as conventional PCR (33), nested-PCR (2), and qualitative real-time PCR (3, 26). These techniques detect DNA from alive and/or dead cells, whereas only conventional methods allowed the selective detection of active cells able to grow on culture medium under laboratory conditions, a procedure that takes around 3 months for identification of *P. chlamydospora* and *P. aleophilum*. The *P. chlamydospora* and *P. aleophilum* TaqMan systems described in this paper allowed the detection of the respective fungi. Identification of *P. chlamydospora* and *P. aleophilum* was also performed following the conventional methods with wood samples to confirm the fungus identification. Both methods gave positive results for all inoculated plants. Conventional methods take around 3 months to yield an estimation of active cells in culture media. The TaqMan systems quantified in a few hours the DNA present in each sample. Abbatecola et al. (1) and Retief et al. (29) used nested PCR to detect *P. chlamydospora* in grapevine rootstocks and in nurseries. However, real-time PCR proved more sensitive, and its results were more reproducible for these species (12) and also for others (21). The advantages of real-time PCR detection over conventional methods are its specificity, sensitivity, and speed. As with all PCR methods, however, the both purifica-

tion of the DNA and primer design influence the efficiency of the reaction.

In the present work, a total of 54 nontarget DNA templates from more than 30 other pathogenic fungi species as well as *Vitis vinifera* were tested to check out specificity of the systems. The detection of *P. chlamydospora* and *P. aleophilum* was consistently specific (100%). In the case of *Pheaoacremonium*, other methods for detection and identification were described previously (3, 26), but only a few other fungal species were included to test specificity. Moreover, this method contributes mostly to the detection of the genera, since the ITS region was not suitable for *Phaeoacremonium* species-specific primer design (26), and degenerated primers were successfully designed for the amplification of nine species of *Phaeoacremonium* (3).

On average, the assays in this work have shown lower C_T values than those in preceding surveys (3, 12, 26); these results suggest higher sensitivity of the primers and probes designed for both *P. chlamydospora* and *P. aleophilum*. Sensitivity of the TaqMan PCR was evaluated using purified DNA and spore suspensions without any DNA purification. The detection limits for purified mycelial DNA were around 100 fg/reaction for *P. chlamydospora* and 50 fg/reaction for *P. aleophilum*. However, 11 fg/reaction of *P. chlamydospora* DNA was detected in 57% of reactions, and 5 fg/reaction of *P. aleophilum* DNA was detected in 23% of reactions. To date, similar sensitivity has been reported only with purified DNA of *Phaeoacremonium parasiticum* (3). Overton et al. (26) and later Edwards et al. (12) could only detect the DNA of *P. chlamydospora* spores after DNA purification. An important result of the present work was the detection of 50 spores in >95% (LOD) of reactions without any DNA extraction step. In the same way, 30 spores (LOD) of *P. aleophilum* were detected with the described TaqMan PCR systems without the need for DNA purification. The results were confirmed with spore suspensions of different isolates of *P. chlamydospora* and *P. aleophilum*.

Among the 30 species associated with grapevine decline, *P. chlamydospora* and *P. aleophilum* represent around 16 to 18% of all isolates identified from grapevine samples analyzed at our laboratory (22). Both species *P. chlamydospora* and *P. aleophilum* are associated with Petri disease and esca (1, 7, 8, 10, 11), although they have also been isolated from plants showing eutypa dieback symptoms (22). To facilitate the diagnosis and taking into account that *P. chlamydospora* and *P. aleophilum* produce spores and mycelia at the same time, wood chip samples were incubated in liquids. These liquids were then used as the DNA template in the TaqMan PCR. At least for *P. chlamydospora*, the TaqMan PCR system provided positive signals in 67% and 42% of the samples incubated in culture medium and water, respectively. To our knowledge, with more or less success, this is the first report of the use of a TaqMan PCR system for *P. chlamydospora* detection in incubation liquid without DNA extraction and without a fungal isolation procedure. For *P. aleophilum*, the TaqMan PCR system only provided a positive signal in 25% of the samples incubated in culture medium; no signal was obtained after water incubation. Differences in detection were obtained depending on the inoculated isolates, although all were 100% detected in wood chips after DNA purification by TaqMan PCR and conventional isolation methods. More investigation is required to confirm these results as well as those obtained with *P. aleophilum*.

The detection methods described here are highly specific, sensitive, and robust enough for the detection of *P. chlamydospora* and *P. aleophilum*. The easy and rapid detection of few spores without DNA purification will be an important advantage to guarantee the pathogen-free status of the propagated material in grapevine nurseries. Because the pchITS439P and PalI1 TaqMan probes designed in this study were manufactured with different reporter dyes at the 5′ end (FAM and HEX, respectively), both species could be detected in the same sample in a duplex reaction, as previously described for some *Phaeocremonium* species (3). The diagnostic methods described here improve on previous techniques and have great potential for use in pathogen-free certification schemes. Detection of *P. chlamydospora* and *P. aleophilum* without the need for fungal isolation reduces analysis times and associated costs. Moreover, the results of this study can be applied to other woody hosts, such as kiwifruit or several *Prunus* species for which *P. aleophilum* has been reported as pathogen.

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